Optical apparatus for multiple points of view three-dimensional microscopy and method

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2013

document version
Publisher's PDF, also known as Version of record

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 November 2013 (28.11.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/176549 A1

(51) International Patent Classification:
G02B 21/22 (2006.01) *G02B 21/36* (2006.01)

(21) International Application Number:
PCT/NL2013/050378

(22) International Filing Date:
24 May 2013 (24.05.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2008873 24 May 2012 (24.05.2012) NL

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: OPTICAL APPARATUS FOR MULTIPLE POINTS OF VIEW THREE-DIMENSIONAL MICROSCOPY AND METHOD

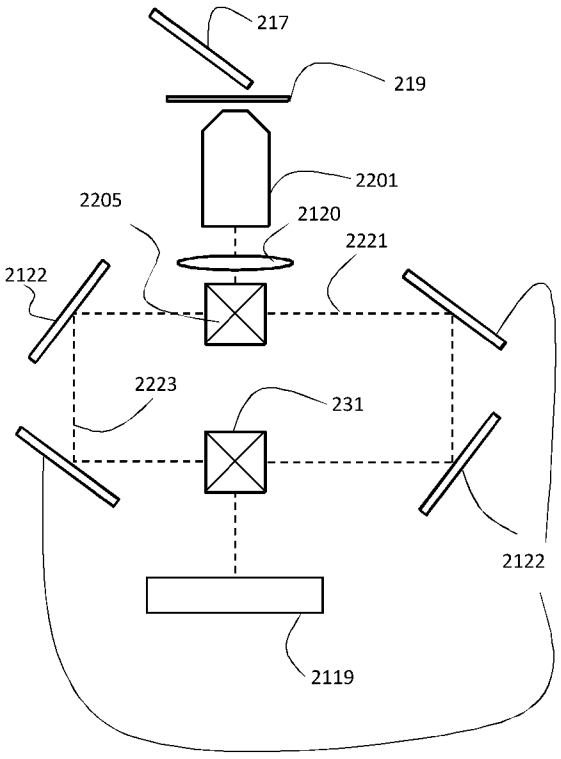


Fig.3

(57) Abstract: The present invention relates to an optical apparatus (202) comprising a sample support (219) and imaging means (2119) arranged, when said optical apparatus (202) is in use, to receive a first beam (2115) emanating from the sample support (219) under a first angle (2127) with respect to a reference axis (2131)wherein the optical apparatus (202) further comprises a reflector (217) and in that the imaging means (2119) are arranged to receive at least a second beam (2113, 211) emanating from the sample support (219) under a second angle (2129) with respect to the reference axis (2131),the second angle (2129) being different from the first angle (2127), the second beam (2113, 211) when passing from the sample support (219) to the imaging means (2119) being reflected by the reflector (217).

WO 2013/176549 A1

Published:

— *with international search report (Art. 21(3))*

OPTICAL APPARATUS FOR MULTIPLE POINTS OF VIEW THREE-DIMENSIONAL MICROSCOPY AND METHOD

The present invention relates to three-dimensional optical microscopy, and particularly to a method and apparatus for three-dimensional optical microscopy with high level of resolution.

Optical microscopy is the tool of choice in medical and biological science. Cell-biology pioneers used straightforward, bright-field microscopy to distinguish cells. Their successors applied more advanced methods such as phase-contrast and differential-interference-contrast microscopy to reveal substructures in cells. Over the last twenty years cell biology has again been revolutionized by fluorescence microscopy in combination with autofluorescent proteins and immunolabeling, which allow localization of biomolecules in cells with high specificity. Localization in three dimensions is achieved by confocal fluorescence microscopy, using one- or two-photon excitation, light sheet based fluorescence microscopy, computational deconvolution, three-dimensional stochastic optical reconstruction microscopy, stimulated emission depletion microscopy and three-dimensional structured illumination microscopy.

In confocal fluorescence microscopy, a focused laser excites a fluorescent sample while fluorescent signal is detected through a pinhole, focused on the same position as the laser, with a sensitive detector. Used in laser-scanning mode, the focused laser beam is scanned point by point through the sample and fluorescence is detected through a pinhole with a photomultiplier or an avalanche photodiode; a two-dimensional image is obtained after a plane of interest has been fully scanned and a three-dimensional volume reconstructed after scanning several of these images. Used in spinning-disk mode, an array of lenses focuses the excitation laser simultaneously in thousands of confocal spots while fluorescence is detected through an array of pinholes focused on these spots with a sensitive camera; a single plane is obtained after rotation of the lens and pinhole arrays and three-dimensional volumes are reconstructed by combining planes obtained with different focus settings. Confocal microscopy suffers from several drawbacks. First, due to the pinholes, which are essential for suppressing out-

of-focus light and enabling sectioning, a substantial amount of fluorescence light is discarded and consequently longer exposure times are required. Second, the depth resolution is at least twofold worse than the lateral resolution. Third, the reconstruction of a three-dimensional volume requires scanning through the sample, which is inherently slow.

With light-sheet based fluorescence microscopy, only a single plane in the sample is excited, by illumination with a thin sheet of light from the direction perpendicular to the optical axis of detection. The key advantage of this technique is that the sample is only excited close to the detection plane, resulting in less background signals and photo-bleaching due to illumination of out-of-focus regions. The spatial resolution of this technique is identical to traditional microscopy, but its sectioning capabilities enable it to reconstruct three-dimensional volume by combining a stack of planes recorded. Again, because it requires scanning through the sample, light-sheet based fluorescence microscopy is inherently slow. Key limitation is that two objectives need to be used perpendicular, with crossing focal planes. This is not compatible with all samples and difficult with high-resolution, short-working distance objectives.

Deconvolution microscopy is a combination of wide-field microscopy with computational deconvolution. Based on the knowledge of the point spread function of the microscope, it extracts information from images taken at different depth to reconstruct the distribution of fluorescent molecules. Results of computational deconvolution depend largely on the sample and on the signal quality, but can surpass confocal microscopy capabilities in some cases. Limitations are that it is an image-processing based method, which requires prior knowledge and can result in artefacts.

Three-dimensional stochastic optical reconstruction microscopy (3D-STORM), stimulated emission depletion microscopy (STED) and three-dimensional structured illumination microscopy (3D-SIM) are three super-resolution techniques enabling to overpass the limit of resolution of conventional microscopy.

3D-STORM is based on wide-field microscopy used with an astigmatic lens and photo-switchable dyes. The basic step in 3D-STORM consists in switching a few fluorophores at a time and to report precisely their three-

dimensional position by fitting. A three dimensional volume is reconstructed from repeating this step hundreds of time. The gain of resolution is substantial, but the volume reported is limited to a few hundreds of nanometer in depth and acquisition of such a volume takes several minutes.

STED is a scanning confocal microscopy technique based: a confocal volume is excited by a first laser while a second laser, used in a doughnut mode, causes stimulated emission of most of the volume except for a small, non diffraction-limited volume. The fluorescence of the molecule in this volume is then detected just like in confocal microscopy. Although the gain of resolution is substantial, the reconstruction of a three-dimensional volume takes at least several minutes for a single cell.

3D-SIM, a development of structured illumination microscopy, consists in reconstituting a two-dimensional image featuring enhanced lateral and axial resolution from several images taken with different illumination structures. A three-dimensional volume is obtained by scanning through the sample. This technique offers double the lateral resolution of a conventional microscope, but its axial resolution is a bit smaller than the one of the invention. Moreover, the fluorescent samples have to stay static while the different illumination structures are projected. Overall this technique is rather slow and cannot by construction access fast features of living organisms.

The methods discussed above are well suited to describe “life at rest” or dynamics occurring at a timescale of about ten of seconds or more with fluorescence microscopy. Faster dynamics, on the second or sub-second timescale are very difficult to follow quantitatively since cells are three-dimensional objects, and scanning the sample in three dimensions is inherently slow and results in poor axial resolution. Except with scanning through the sample at high rate, these techniques only provide access to motion in the focal plane and the axial component is lacking. High rate scanning is not a solution either, since it leads to an important loss of sensitivity. In studies of processes in single cells or cells in living, multicellular organisms, this issue makes quantitative imaging of dynamics on the sub-second scale hardly possible.

Thus there is a need for a method and apparatus for three-dimensional optical microscopy which provides live three-dimensional information, which

has an enhanced axial resolution and which is not limited to fluorescence microscopy, but provides live three-dimensional information in bright field microscopy, dark-field microscopy, phase-contrast microscopy and differential-interference-contrast microscopy.

The present invention satisfies these needs, as well as others, and generally overcomes the deficiencies found in known optical microscopy devices and methods.

An object of the invention is to provide a method and apparatus for three-dimensional microscopy which provides live three-dimensional information and live three-dimensional recording.

Another object of the invention is to provide a method and apparatus for three-dimensional microscopy which provides an enhanced axial resolution.

Another object of the invention is to provide a method and apparatus for three-dimensional microscopy which enables live three-dimensional observation and live three-dimensional recording of thick specimens

Another object of the invention is to provide a method and apparatus for three-dimensional microscopy which allows observation of an object under any desired angle with bright-field microscopy, dark-field microscopy, phase-contrast microscopy and differential-interference-contrast microscopy.

Another object of the invention is to provide a method and apparatus for three-dimensional microscopy which allows the three-dimensional reconstruction of an object with enhanced resolution.

Another object of the invention is to provide a method and apparatus for three-dimensional microscopy which provides confocal microscopy with enhanced resolution.

The above objectives are achieved by the following optical apparatus and method.

Optical apparatus comprising

a sample support and

imaging means arranged, when said optical apparatus is in use, to receive

a first beam emanating from the sample support under a first angle with respect to a reference axis

wherein the optical apparatus further comprises

a reflector and in that the imaging means are arranged to receive at least

a second beam emanating from the sample support under a second angle with respect to the reference axis,

the second angle being different from the first angle, the second beam when passing from the sample support to the imaging means being reflected by the reflector.

Optical method to image a sample using an imaging means which use a first beam emanating from said sample under a first angle with respect to a reference axis and a second beam emanating from said sample under a second angle with respect to said reference axis to image the sample and wherein this second angle is different than the first angle and wherein the second beam is reflected when passing from the sample to the imaging means.

Preferably the method according to the invention is performed in the optical apparatus according to the invention.

Applicants found that by using the reflected second beam, some of the light emitted in a different direction can actually be collected and this light can be used to image the sample simultaneously under two different angles. The images taken are limited by their depth of field, but where the images allows the observation of the same part of the sample under different angles, it enables one to obtain a three-dimensional image of this sample part, even with specimen thicker than typically one micrometer, without having to perform a "scanning step". The optical apparatus and method therefore enables one to image a sample much quicker than techniques known in the prior art. Applicants found that a higher axial resolution can be achieved, sometimes even two times higher, than when using conventional microscopy. Applicants found that by increasing the depth of field of the sample images taken under different angles, it enables one to obtain a three-dimensional image of specimen thicker than several times the axial resolution of the microscope objective used, typically several micrometers for the microscope objectives of very high numerical aperture, without having to perform a "scanning step"

The optical apparatus and method has been found to provide access to for example three-dimensional information at high resolution from

fluorescence microscopy, dark-field microscopy, phase-contrast microscopy, differential-interference-contrast microscopy and confocal microscopy. This makes the current apparatus and method unique as it is not known to applicants that such a feature is achievable by any kind of the known microscopy techniques.

Further objects and advantages of the invention will be brought out in the following portions of the specification, wherein the detailed description is the purpose of fully disclosing preferred embodiments of the invention without placing limitations thereon.

The optical apparatus may further comprise a beam splitter arranged to receive the first beam and the second beam and to split the first beam and the second beam between a first path and at least a different second path,

The optical apparatus may further comprise a beam combiner arranged to combine the first and second beam and direct the combined beam to the imaging means. The apparatus may further comprise means for guiding said first path such as for example mirrors and optical fibres. The beam splitter and beam combiner may be one beam splitting and recombining means. Examples of such beam splitter and beam combiner means are a polarizing beamsplitting cube, a polarizing plate beamsplitter, a non polarizing cube beam splitter, a beam sampler, a dichroic mirror or a semi reflective mirror.

The optical apparatus may further comprise a main objective lens. The sample support may be positioned between the objective lens and the reflector.

The optical apparatus may further comprises in the path of the first and/or second beam a far focusing reflector, and a quarter-wave plate and a beam splitter means positioned before the far focusing reflector. The far focusing reflector may optionally be combined with a far focusing objective lens wherein the far focusing reflector faces the front lens of said far focusing objective lens. If a far focusing objective lens is present the beam splitter will be positioned before such a far focusing objective. Combinations can be envisaged with one beam, a far focussing objective lens and a far focusing reflector, two beams each provided with a far focussing objective lens and a far focusing reflector and two beams wherein one beam is provided with a far focussing objective lens and a far focusing reflector and the other beam is

provided with a far focusing reflector only. Suitably such a combination is present in the path of the first and second beam. Such a far focusing objective lens and a far focusing reflector is advantageous because it allows the user to focus in a plane in the sample which is not accessible by conventional microscopy.

The optical apparatus may further comprises rotation means to rotate the quarter-wave plate. Such a means to rotate the quarter-wave plate is advantageous because it allows the user to tune the polarization of the light before it enters and after it exits the combination of the far focusing and the far focusing reflector.

The optical apparatus may further comprise in the path of the first and/or second beam a spatial filter to spatially filter light coming from said far focusing objective. Such a spatial filter may be advantageous because they allow the user to easily separate the light coming from the first and second beam when they are combined later on an imaging means. Applicants found that the apparatus may also function adequately without using such a spatial filter.

The optical apparatus may further comprise in the path of the first and second beam one or more lenses to conjugate appropriately the image of the sample toward the far focusing objective lens.

The imaging means for detecting and recording images may be positioned to detect and record the observed light, wherein the observed light is the light which has been split by beam splitting and recombining means, far focused by the far focusing objective lens or lenses, filtered by the spatial filter or filters and combined by the beam splitting and recombining means. The imaging means may be a camera with an array of sensitive detectors or point detectors and will depend on the type of microscopy.

The optical apparatus may further comprise a plurality of means to direct light from the sample support to the beam splitting and recombining means as well to and from the one or two far focusing objective lenses and towards the imaging means. Such means to direct light may be mirrors, prisms, optical fibers or array of optical fibers.

The optical apparatus may further comprise means to visualize the said microscope sample. Examples of means to visualise are imaging lenses optionally combined with a camera.

The optical apparatus may further comprise means to adjust position and orientation of the reflector relative to the sample support. For example means to rotate the sample support relative to the reflector may be present. Suitably the apparatus further comprises means for sensing position and orientation of the reflector relative to the sample support. This is advantageous because it allows the user to select precisely the position and orientation where to image the sample.

The optical apparatus may comprise means to adjust position of said main objective lens relative to the sample support. Such means are well known and may be for example a microscope stage, an electrical or mechanical translation actuator, a microscope objective scanner. This is advantageous because it allow the user to focus at any depth desired in the sample.

The optical apparatus may further comprise means for sensing position of the main objective lens relative to the sample support. Such means are well known and may be for example a capacitive sensor or a gauge sensor. This is advantageous because it allows the user to know where the focus is achieved in the sample. The means to adjust position is responsive to the means for sensing position.

The optical apparatus may further comprise means to adjust the position of the spatial filter in the path of the first and/or second beam. Such means are well known and may be for example a translation stage. This is advantageous because it enables the precise selection of each path field of view.

The optical apparatus may further comprise means to adjust position of the far focusing objective lens relative to the far focusing reflector. Such means are well known and may be for example a translation stage or a piezo electric translator. This is advantageous because it allows the user to select the depth at which refocusing is achieved.

The optical apparatus may further comprise means to adjust position of the far focusing reflector relative to the far focusing objective lens. Such

means are well known and may be for example a translation stage or a piezo electric translator. Suitably the apparatus also comprises means for sensing position of the far focusing reflector relative to the far focusing objective lens. Such means are well known and may be for example a capacitive sensor or a gauge sensor. Suitably the means to adjust position is responsive to the means for sensing position.

The optical apparatus may further comprise sample illumination means for providing illuminating light to the sample support. The illuminating light is positioned such as to provide illuminating light to the main objective lens. Such means are well known and may be for example a white lamp, LED lamp, other extended spatially incoherent light source or lasers and will depend on the type of microscopy.

The optical apparatus may further comprise sample side illumination means for providing side illuminating light to the sample support. The side illuminating light may be positioned such as to provide illuminating light to the reflector and the sample support. Such means are well known and may be for example a white lamp, LED lamp, other extended spatially incoherent light source or lasers. This is advantageous because it provides illumination light under angles which are not usually accessible to conventional illumination systems.

The optical apparatus may further comprise selective transmittance and reflectance means for transmitting the light from the main objective lens toward the imaging means and reflecting said illuminating light away from said imaging means. Such means are well known and may be for example dichroic mirrors. This is advantageous because it allows excitation light and emission light to be separated when achieving fluorescence microscopy or more generally to separate different light wavelengths that would need to be treated separately.

The optical apparatus may further comprise filtering means for transmitting the observed light from the main objective lens and filtering the illuminating light from the illuminating means. Such means are well known and may be for example emission filters. This is advantageous because it selects the light coming from the emitting molecules as usually done in fluorescence microscopy.

The optical apparatus may further comprise selective transmittance and reflectance means for reflecting the observed light from the main objective lens toward the imaging means and transmitting the illuminating light away from said imaging means. Such means are well known and may be for example dichroic mirrors. This is advantageous because it allows different light wavelengths to be spatially separated when necessary.

The reflector may be a planar mirror. A suitable reflector is a beam divider, suitably a beam divider composed of a glass substrate optionally presenting a repeated structure and optionally coated with a dielectric or a metallic layer. The reflector may also be a substrate presenting a single dihedral structure. Suitably such a reflector is composed of a glass or of a plastic substrate, for example a soft or stiff plastic substrate presenting a repeated structure. The reflector may be optionally coated with a dielectric or a metallic layer. The reflector may be made of for example poly(methyl methacrylate) (PMMA), poly(dimethylsiloxane) (PDMS) or silicon. More preferably the substrate comprises more than one dihedral structure, which can cross and whose crossing orientation in respect to each other can range from 0 to 90 degrees, the preferred orientation depending on the application.

The optical apparatus may further comprise a microfluidic device comprising at least one flow channel obtained by standard soft lithography with a polymer layer, preferably made of low refractive index optical adhesives or coating materials, or poly(dimethylsiloxane) (PDMS). This flow channel may be used to ease the sample preparation and may be combined with the reflector described above.

The optical apparatus may further comprise active or passive means to modify phase and amplitude of the light emanating from the sample. Example of active means are liquid crystal arrays or digital micromirror device. Example of passive means are phase plates, spatial filters, pinhole or axicon. This is advantageous because it allows specialized microscopy such as differential interference microscopy, phase contrast microscopy, dark field microscopy, confocal microscopy and extended depth of field to be achieved. Preferably means for positioning these active or passive means are present. This is advantageous because the active and passive means to modify phase and

amplitude are very sensitive to position and fine tuning of this position is therefore advantageous.

The invention is also directed to the below methods which may be performed with the above described apparatus wherein the sample is positioned in the sample support. The method comprises the steps of:

(a) focusing said the main objective lens about the said microscope sample;

(b) oscillating the focus of the far focusing objective lenses upon a volume within one of the images of the said sample;

(c) directing the images observed by said far focusing objectives to the image detection means for image recording.

This method is advantageous because it enables the user to extend significantly the field of view of the images recorded. Since two or more orientations of the same object are obtained simultaneously with an extended depth of field, the user is able to observe in live and in three dimensions a much larger volume than would allow conventional microscopy.

The method may also comprise the steps of:

(a) focusing said the main objective lens about the said microscope sample;

(b) focusing the far focusing objective lenses upon a section or plane within the image of the said sample;

(c) positioning adequately the active or passive means to modify phase and amplitude of the observed light in order to achieve dark field microscopy, phase microscopy, differential interference contrast microscopy, confocal microscopy or to extend the depth of field of the image of the said sample.

This method is advantageous because it enables to extend the depth of field of the microscope with the advantages described in the method above, and it also enables dark field microscopy, phase microscopy, differential interference contrast microscopy to be performed and to observe simultaneously at least two independent sections of the same sample with one or a combination of these techniques. Furthermore, this method is advantageous, because it enables confocal microscopy to be performed with

the excitation and the detection taken from different angles, thereby increasing the axial resolution of this kind of microscopy.

The present invention generally pertains to a method and apparatus for three dimensional optical microscopy which employs an objective and a reflector about a sample and one or two additional objectives to achieve far focusing.

The present invention generally pertains a method and apparatus for three dimensional optical microscopy which employs an objective and a reflector about a sample and one or two additional objectives to achieve far focusing.

There are four preferred embodiments of the invention which, employing essentially the same apparatus, allow imaging a sample with two or more points of view thanks to one or more reflectors about the sample and far focusing achieved by one or two additional objectives.

By way of example and not of limitation, the present invention generally includes an objective lens and a reflector which are mounted about a sample, the reflector being preferably in the form of a mirror or a faceted mirror, with the reflector mount including translation and rotation means. For far focusing means, the invention generally includes one or two extra objective lenses combined with one mirror and one quarter-wave plate each, objectives and mirrors being preferably mounted with translation and/or rotation means. Illumination means, preferably in the form of one or more LED lamps or other extended spatially incoherent light source and one or more lasers, provides illumination for the sample. The invention shall be illustrated below by means of illustrative embodiments and figures. It will be clear to the skilled person that other combinations of the parts of the optical apparatus according to the invention which may offer the same advantages can also be envisaged by the skilled person.

The invention preferably generally includes beam splitter and combiner means, preferably in the form of beam splitter-recombiner cubes, for splitting and combining the light coming from the sample for recording. A plurality of adjustable mirrors allows the direction of illuminating and/or observed light to and from the objective lenses and image recording means. The image recording means preferably includes a CCD or EMCCD camera. Means for

selectively transmitting and reflecting light of different wavelengths, preferably in the form of dichroic mirrors and/or optical filters, are generally included in the invention. Optical path length adjustments means preferably in the form of a translating stage with one or more suitably positioned mirrors, allows tuning of the optical path lengths. Phase and/or amplitude alteration means, preferably in the form of a spatial light modulator or phase plates, may be included for alteration of the phase and/or amplitude of the illumination and observed or emitted light. Alignment means for positioning the sample relative to the objectives lenses are provided, which preferably includes a translational stage or a translational-rotational stage, an objective and/or lenses combined with a CCD camera including translation means. The invention may employ vibration and/or light isolation supporting means such as vibration isolated platform or housing.

In a first embodiment of the present invention, the reflector, which is mounted about the sample, creates one or more mirror images of the sample. Illuminating light is generally directed to the sample from the objective lens or from the side of the sample. The objective lens is used to image the volume of the sample and the images of this volume. The light coming from the objective is split and then projected by the far focusing objectives. A plane of observation is selected in these far focusing objectives by the mean of a mirror. The images selected by the far focusing objective lenses are spatially filtered and then combined on a CCD camera or other imaging means. This first embodiment applies primarily to fluorescence, phosphorescence and bright-field microscopy.

In a second embodiment of the invention, the first embodiment is combined with Fourier optics to generate dark-field, phase-contrast and differential-interference-contrast multiple points of view microscopy. To this mean, the images created by the far focusing objective lenses are combined and filtered in amplitude and/or phase with a passive or active spatial light modulator. The Fourier conjugated planes of these images can be used to apply this filtering. Different amplitudes and/or phases are used to achieve dark-field, phase-contrast or differential-interference-contrast multiple points of view microscopy by finally conjugating the images on a CCD camera or other imaging means. Preferably the optical apparatus comprises a beam combiner

arranged to combine the first and second beam and direct the combined beam to the imaging means. In the path of the combined beam to the imaging means a spatial light modulator and two lenses a and b are positioned. Lens a is positioned so that its back focal plane coincides with the far focusing objective back focal plane. The spatial light modulator is positioned in lens a front focal plane. The lens b back focal plane is positioned on the spatial light modulator to image the two different planes selected by the two far focusing objective lenses side by side on the imaging means. Instead of the referred to spatial light modulator any other "active or passive means to modify phase and amplitude" may be used.

In a third embodiment of the invention, which applies primarily to fluorescence and phosphorescence microscopy, the first embodiment is combined with computational means to reconstruct a three-dimensional image of the sample. For this mean, the volume is scanned by one or more of the far focusing objectives, and volumes observed under different angles are recorded. The three-dimensional reconstruction consists in combining numerically the information in these volumes. Reconstruction is achieved by comparing the volumes in the real space, such as, but not limited to, using the minimum of intensity in each position of the three-dimensional space, or by combining the information of each volume in the Fourier space and then inverting this information, to create a reconstruction of the volume with sectioning and increased resolution.

In a fourth embodiment of the invention, the first embodiment is combined with confocal excitation and detection to reconstruct a three-dimensional image of the sample. The excitation is provided by focusing light through the objective close to the sample while detection uses the mirror image of the sample, enabling detection with an angle to the excitation axis. Detection is achieved by recovering the fluorescence signal by one of the far focusing objectives, selecting the mirror image of the excitation spot created by the mirror close to the sample, and imaging it on a pinhole before it is detected by an avalanche photodiode or other detection means.

The invention will be more fully understood by reference to the following drawings, which are for illustrative purpose only:

FIG. 1 is a schematic diagram of an embodiment of an optical microscope in accordance to the present invention.

FIG. 3 is a schematic diagram of an embodiment of an optical microscope in accordance to the present invention.

FIG. 3 is a schematic diagram of an embodiment of an optical microscope in accordance to the present invention.

FIG. 4 is a schematic diagram of a first embodiment of an optical microscope in accordance to the present invention.

FIG. 5 is a schematic diagram of the first embodiment of an optical microscope including an illumination mean.

FIG. 6 is a schematic diagram of the first embodiment of an optical microscope including another illumination mean.

FIG. 7 is a schematic diagram of a reflector mean to be used in the sample plane of the first embodiment of an optical microscope.

FIG. 8 is a schematic diagram of another reflector mean to be used in the sample plane of the first embodiment of an optical microscope.

FIG. 9 is a schematic diagram of another reflector mean to be used in the sample plane of the first embodiment of an optical microscope.

FIG. 10 is a schematic diagram of a hybrid microfluidic reflector device to be used in the sample plane of the first embodiment of an optical microscope.

FIG. 11 is a schematic diagram of visualization mean to be used in an optical microscope in accordance to the present invention.

FIG. 12 is a schematic diagram of the first embodiment of an optical microscope with additional flipping mirrors to provide traditional wide-field and epi-fluorescence microscopy.

FIG. 13 is a schematic diagram of a second embodiment of an optical microscope in accordance to the present invention.

FIG. 14 is a flow diagram showing the general steps of a third embodiment of an optical microscope in accordance to the present invention.

FIG. 15 is a graphic representation of the object and its mirror image in the sample plane 11a, where only a single plane reflector 10 is used.

FIG. 16 is a flow diagram showing the general steps of a method based on volume comparison to reconstruct a three dimensional volume from a data set combining image recordings of a volume under several directions.

FIG. 17 is a simplified graphic representation of the iso-intensity (half maximum intensity, in x-z plane, where z is the optical axis of the main objective and x, any axis in the observation plane) of the light emitted by a point source and detected by in a conventional fluorescence microscope.

FIG. 18 is a simplified graphic representation of the iso-intensity (half maximum intensity, in x-z plane, where z is the optical axis of the main objective and x, any axis in the observation plane) of the light emitted by a point source, detected and processed by using the apparatus and method of the third embodiment.

FIG. 19 is a flow diagram showing the general steps of a method based on three-dimensional Fourier information to reconstruct a three dimensional volume from a data set combining image recordings of a volume under several directions.

FIG. 20 is a schematic diagram of a fourth embodiment of an optical microscope in accordance to the present invention.

FIG. 21 is a simplified graphic representation of the excitation light in a conventional confocal microscope.

FIG. 22 is a simplified graphic representation of the excitation light reflected on the reflector 10 used in the apparatus 170.

FIG.23 is a simplified graphic representation of the detection method to be used with the apparatus 170.

Detailed Description of the Invention

Referring more specifically to the drawings, for illustrative purposes, the method and apparatus comprising the present invention and the underlying theory behind the invention are generally shown in FIG 1 through FIG 23.

It will be appreciated that the apparatus of the invention may vary as to configuration and as to details of the parts and that the method of the invention may vary as to the steps and their sequence, without departing from the basic concepts as disclosed herein.

Fig. 1 shows an optical apparatus 202, a sample support 219 and imaging means 2119. In use imaging means 2119 will receive a first beam

2115 emanating from the sample support 219 under a first angle 2127 with respect to a reference axis 2131. Also shown is a reflector 217. The imaging means 2119 will also receive a second beam 2113, 211 via the reflector 217 emanating from the sample support 219. The light emanating from the sample support is emanated under a second angle 2129 with respect to the reference axis 2131. The second angle 2129 is different from the first angle 2127. The second beam 2113, 211 when passing from the sample support 219 to the imaging means 2119 is reflected by the reflector 217.

A sample that is under study in an apparatus according to Fig. 1 will radiate light in a lot of different directions, i.e. under a lot of different angles. Prior art 3D microscopes are only able to image light emitted in one direction and 3D reconstruction is achieved by performing what we will refer to as a "scanning step", that is light emitted at a given focus is imaged, the position of the sample relative to the microscope objective is changed and light emitted at different focus is imaged. This technique is relatively slow and not suitable for instance to image 3D samples (e.g. living organisms) which are not at rest and for which the ability for a quick image is paramount.

The invention is based on the realization that by using a reflector 217, some of the light emitted in a different direction can actually be collected and this light can be used to image the sample simultaneously under two different angles. As no "scanning step" is needed, this technique is therefore much quicker than techniques known in the prior art.

It should be noted that the light emitted in the direction 2117 would without the presence of the reflector 217 not be available for imaging.

The images can for instance be obtained by suitable detectors. A first detector 2119 can simply be placed in the path of the beam of light 2115. The location for a second detector 2121 can be obtained by constructing a virtual image 213 of the sample 219. This is done by mirroring (angles alpha and beta in figure 21 being equal) the sample and the second beam of light 2113 in a line 2123 passing through the reflector 217. A suitable location for the second detector 2121 is on the prolongation of the virtual image of the second beam of light 2113. The path traced by the beam of light emitted in the second direction 2117 is then from the sample 219 to the reflector 217 (path taken by

the second beam of light 2113 and from the reflector 217 in the direction referenced 211 to the second detector 2121.

The reflector 217 thus permits to image the sample 219 under two different angles, namely under an angle 2127 formed by the first beam of light 2111 and the surface of the sample 219 and an angle 2129 formed by the second beam of light 2113 and the surface of the sample 219.

In Figure 2 an optical apparatus is shown where to he apparatus of Fig. 1 is a primary lens 2201, a beam splitter 2205, two lenses 2203, 2207 and two imaging means 2119 are present. The beam splitter 2205 is arranged to receive the first beam 2115 and the second beam 2113, 211 and to split the first beam 2115 and the second beam 2113, 211 between a first path 2221 and at least a different second path 2223. This apparatus is advantageous over the apparatus of Figure 1 in that light coming from the sample is relayed through the lenses, giving much better quality images and that the two paths enable imaging at two different focuses simultaneously.

Figure 3 shows an apparatus where the light emitted from the primary lens 2201, a tube lens 2120 and a beam splitter 2205. The resulting first 2223 and second 2221 beams are directed such that they meet again in a beam combiner 231 arranged to combine the first and second beam and direct the combined beam to the imaging means 2119. Figure 3 also shows 4 mirrors 2122 as the guiding means for the two beams of light 2223, 2221. This is advantageous because it requires only one imaging means and means for guiding said first path to be used in the apparatus.

FIG. 4 discloses a simplified schematic diagram of the microscope apparatus 1 in accordance with a first embodiment of the present invention: An objective 16 and a reflector 10 are mounted about a sample 11a. The reflector 10 may be of different forms and is discussed below. The objective 16 is focused on the sample while the reflector 10 is brought close to the sample and reflects the object in the sample. The sample 11a is preferably mounted on a coverglass or in a microfluidics chamber including a reflector (discussed below). Generally, illuminating light for illuminating means is directed to the sample 11a from the side of the sample, partially reflected on reflector 10 or through the objective lens 16 using a beam splitter, which may or may not be dichroic, as will be discussed below. The observed light or

images from objective 16 is reflected by a mirror 12a, and then split by a beam splitter, preferably a polarizing cube 14a. The observed light or images from objective 16 are then directed to the objective lenses 17a and 17b for far focusing purpose along paths 19. Along path 19a, light goes through cube 14b and a quarter-wave plate 15a and enters objective lens 17a. Lenses 13a and 13b are placed to ensure that the back focal plane of objective 16 is conjugated with the back focal plane of objective 17a. The translation and rotation of mirror 12b enables to select a plane of observation in the sample 11b duplicated from the sample 11a. When the light exits the objective 17a, it goes through the quarter-wave plate 15a for a second time. The quarter-wave plate 15a is oriented, so that the polarization of the light passed twice through it undergoes a 90 degrees rotation. The light is therefore reflected by polarizing cube 14b. The lens 13c creates an image of the plane selected with objective 17a and mirror 12b on the plane 18a, where a spatial filter, preferably a razor blade or a mechanical slit, is preferably positioned. The light is then reflected by mirrors 12c, 12d, 12e, 12f and cubes 14a and 14b to be imaged on image detector 20. Lenses 13d and 13g are placed to ensure that the spatial filter 18a is conjugated with the image detector 20 plane. Preferably, image detection means 20 is a CCD camera or the like, and it may or may not be preceded by dichroic or filtering means (not shown) especially when used in fluorescence or phosphorescence microscopy. The light going along path 19b undergoes similar transformations as the light going along path 19a, with the difference that the polarization of path 19a is 90 degrees rotated compared to path 19b.

In the operation of the microscope apparatus 1, two different planes to observe in the sample 11a are selected by objective lenses 17 by positioning mirrors 12b and 12g. These planes are then imaged side by side on the image detector 20. In order to avoid image mixing on the image detection means 20, spatial filters on planes 18 are positioned to select the part of interest in the image while mirrors 12f and 12j are placed and can be tilted in the back focal plane of lens 13g to translate the two images obtained from objective lenses 17a and 17b respectively. Fully flexible imaging of the sample 11a may be ensured by mounting it on a translation stage or a translation rotation stage

(not shown), as well as mirror 10 may be mounted on a stage with translation and rotation means (not shown).

Several arrangements of mirrors 12 and lenses 13 may be employed for the microscope apparatus 1, but they should satisfy the conjugation relations defined above.

FIG. 5 shows generally a possible way to illuminate the sample 11a. Illuminating light for illuminating means can be directed to the sample 11a through the objective lens 16 using a beam splitter 23, which may or may not be dichroic. The light source 21, which may or may not be a spatially incoherent light source, may or may not be collimated by a lens 22 into the back focal plane of the objective lens 23.

FIG. 6 shows generally another possible way to illuminate the sample 11a. Illuminating light for illuminating means can be directed to the sample 11a from the side of the sample 11a. The light source 30, which may or may not be a spatially incoherent light source, may or may not be collimated by a lens 31 onto the sample 11a and the mirror 10.

FIG. 7 shows generally a type of reflector 40 applicable in the sample 11a. The reflector 40 consists of a plane mirror, preferably is a dielectric mirror, coated metallic mirror or the like. There is no limitation to the shape or size of the mirror 40.

FIG. 8 shows generally a type of reflector 50 applicable in the sample 11a. The reflector 50 is a beam divider, preferably glass, plastic or crystalline substrate presenting a repeated structure, coated with a dielectric or a metallic layer. The length of repetition of the dihedral structure “a+b” is in the range from 1 micrometer to 5 millimeters, where “a” and “b” might or might not be equal. The angle α defining the orientation of the structure’s facets is in the range from 45 to 180 degrees. Although intended to be tiltable, reflector 50 is preferably oriented with the main dihedral axis perpendicular to the optical axis of the objective lens 16.

FIG. 9 shows generally a type of reflector 60 applicable in the sample 11a. The reflector 60 is a geometrical simplification of beam divider 50, where, all others geometrical and physical properties conserved, only one dihedral structure is present. Several arrangements of these structures are possible, differing in their number, orientation or geometry on the same reflector.

FIG. 10 shows generally the reflector 73 embedded in a microfluidic apparatus 70. The reflector 73 is preferably one of the reflectors 50 and 60 presented above. The creation of channel 75 and the embedding of the reflector 73 are obtained by standard soft lithography with a polymer layer 74, preferably made of low refractive index optical adhesives or coating materials, or poly(dimethylsiloxane) (PDMS). The polymer layer 74, with the embedded reflector 73, is coated on a coverslip 72. Tubing 71 ensure the flow circulation in the channel 75.

FIG. 11 shows generally a possible additional visualization apparatus 80 to the microscope apparatus 1. With the reflector 10 mounted with translation means, it can be translated to enable visualization from the top of the sample 11a. A lens 81, preferably a lens or an objective lens is collimated on sample 11a. The sample 11a is then imaged on the image detection means 82, which is preferably a CCD camera or the like. FIG. 8 shows the simplest configuration for visualization, but additional lenses or mirrors may be inserted. An ocular may also be used instead of image detection means 82. Lens 81 and image detection means 82 may be mounted on translational means to achieve proper conjugation with the sample.

FIG. 12 shows generally a possible apparatus variation 90 of the microscope apparatus 1. Mirrors 91 may be inserted in the path 93a to shortcut the far focusing paths 19a and 19b, and to direct the light toward the image detection means 20. It enables the microscope to be used as in a traditional wide-field or epi-fluorescence microscope. The mirrors 91 are preferably mounted on translation or flipping means to ensure adequate operation between traditional and multiple points of view visualization. The lens 92 is suitably positioned to image the sample on the image detection means 20. FIG. 9 shows the simplest configuration for traditional visualization, but additional lenses or mirrors may be inserted.

Referring to FIG. 13, a simplified schematic diagram of the microscope apparatus 100 in accordance with the second embodiment of the present invention is generally shown. Path 19c of apparatus 1 is modified to combine multiple points of view microscopy with Fourier filtering optics. Lens 101a is positioned so that its back focal plane coincides with lens 13g front focal plane. In case the apparatus is simplified such as there is no optics between beam

splitter 14a and refocusing objective 17a, these two lenses 101a and 13g may be replaced by lens a, as described above when discussing the second embodiment. The spatial light modulator (SLM) 103 is positioned in lens 101a front focal plane. Lens 101b (lens b) back focal plane is positioned on the SLM to image the two different planes selected by objective lenses 17 side by side on the image detection means 104. Preferably, image detection means 104 is a CCD camera, EMCCD camera or the like.

In the operation of the microscope apparatus 100, different amplitude and/or phase can be applied on the SLM 103 to achieve the type of microscopy intended as is known to the skilled person. The selection of observed planes in the sample 11a is achieved as in the operation of the microscope apparatus 1. Therefore, the apparatus 100 enables dark-field microscopy, phase-contrast microscopy and differential-interference-contrast microscopy to be used and to observe the sample 11a under any angle with these types of microscopy. The apparatus 100 of Figure 13 may also be used to extend the depth of field of the image when used to observe the sample 11a.

Preferably the above method according to the invention is performed in the optical apparatus according to the invention. The invention is also directed to the following method as performed in this optical apparatus. A method for three-dimensional optical microscopy of a microscope sample comprising the steps of:

(a) focusing said a main objective lens about the said microscope sample;

(b) focusing far focusing objective lenses upon a section or plane within the image of the said sample;

(c) directing images observed by said far focusing objectives to an image detection means for image recording;

(d) adjusting said far focusing mirrors facing said far focusing objectives to enable imaging of a new set of volume sections;

(e) repeating steps (a), (b), (c) and (d) until a plurality of section of said sample has been observed and recorded, forming a data set of recorded images;

(f) processing data set obtained at step (e) to extract the images of the said microscope sample obtained with different orientations;

(g) reorienting the said images obtained at step (f) in the same base;

(h) combining the images obtained at step (g) to create a new volume image of the sample with enhanced resolution.

Referring now to FIG. 14, a simplified flow chart relating the above three-dimensional reconstruction method and also in accordance with the third embodiment of the present invention is generally shown. The following description takes into account the operation of both far focusing objective lenses 17. The operation can be simplified by using only one of these objectives, but it may require longer acquisition time; the reconstruction part is however not changed.

At step 111, the objective lens 16 is focused about the sample. Focusing is preferably carried out by moving the objective lens, or the sample, on precision translating means such as translating stages.

At step 112, far focusing objective lenses 17 are focused upon a section or plane within the sample. Focusing is preferably carried out by moving the objective lenses, or the sample, on precision translating means such as translating stages.

At step 113, the images observed by both far focusing objectives are directed to image detection means such as a CCD camera or the like for image recording.

At step 114, mirrors facing far focusing objectives are adjusted to enable imaging of a new set of volume sections. These adjustments are intended to provide volume sections whom distance from sections obtained at step 112 is preferably half of the lateral spatial resolution of one image (i.e. typically 100 nm).

At step 115, steps 113 and 114 are repeated, until each section of the sample has been observed and recorded as related above. The recorded images from each section of sample, under two or more orientations, form a data set for the entire sample, which is stored by a microprocessor interfaced to the image detection means.

At step 116, the data set obtained at step 115 is processed a first time. As shown on figure 12, the data set 120 obtained at step 115, consists of the

record of the sample duplicated by mirror transformations. The sample 121 is duplicated here, as a matter of example, in the mirror image 122. One or more than one of these mirror transformations are preferably intended to be used in the invention; the following explanations are limited to one mirror image, but it can be easily generalized to more than one of these mirror images. Each mirror volume image is extracted from the data set, enabling independent transformations of these volumes in reduced data sets for the next steps.

At step 117, the sample and its mirror images are reoriented in the same base. As an example, following the schematic proposed on FIG. 15, the mirror transformation which gives the image 122 of the sample 121 has to be inversed to combine the sample and its image. For this purpose, different options can be taken. A first option is to use a direct mirror transformation through a matrix operation addressing each voxel (three-dimensional pixel) in the mirror image obtained at step 115 to a new voxel or a new set of voxels in the volume created by the inverse mirror transformation. The intensity value of each addressed voxel may be interpolated from neighbouring source voxels to enable adapted addressing. Another option is to transform the volume in the Fourier space: the volume is first Fourier transformed in three-dimension; it undergoes several transformations necessary to perform the desired final transformation (combination of rotations, translations or planar symmetry can be considered). The advantage of this latter technique is that it enables subpixel transformation. In each case the transformation characteristics can be determined beforehand through calibration or by volume comparison.

At step 118, the sample and its mirror images are combined to create a new volume image of the sample with enhanced resolution. Several methods can be used; as a matter of example, two are proposed. The first method is generally presented in FIG. 16. As the sample and its mirror images have been reoriented in step 117, these volumes can be compared. At step 131, the average intensity of each volume is first equalized, because of possible losses which might have happened while recording the data set. At step 132, the voxel at the same exact position in each volume is examined and at step 133 the corresponding intensities are compared. The voxel of the new volume image at this given position is assigned with the minimum of the intensities compared. At step 134, steps 132 and 133 are repeated to assign the

intensity value of each voxel in the new volume image. The result of imaging a single fluorescent point in a conventional microscope is shown on FIG. 17; the graph represents the iso-intensity of half maximum intensity in x-z plane, where z is the optical axis of the main objective and x, any axis in the observation plane. The result of imaging a single fluorescent point with the method described in FIG. 13 is shown on FIG. 18; the reflector used is supposed to be the one of the reflector described in FIG. 5 or 6, where the angle α is 60 degrees; the graph (plain line) represents the iso-intensity of half the maximum intensity in the x-z plane, where z is the optical axis of the main objective and x define the x-z plane as perpendicular to the planes of the reflectors. The image resulting from the method described in FIG. 13 has an enhanced axial resolution.

The second method for performing step 118 is generally presented in FIG. 19. At step 161, each volume obtained at step 117 is first Fourier transformed. One obtains the three-dimensional Fourier transform of each volume. At step 162, these Fourier transforms are combined in Wiener filter. At step 163, the result of step 162 is Fourier inversed to obtain the complete reconstructed volume.

The advantage of the following embodiment is that one could in principle go deeper in the sample.

Referring to FIG. 20, a simplified schematic diagram of the microscope apparatus 170 in accordance with the fourth embodiment of the present invention is generally shown. The apparatus 170 is a modification of the apparatus 1 enabling confocal excitation of the sample 11a and confocal detection. The light source 174, preferably a spatially coherent light source, is focused by a lens 171 on a scanning device 172, preferably a scanning galvanometer mirror system. The light is then directed to the objective 16, as it is collimated by lens 173 and reflected on the dichroic mirror 177. The objective lens 16 converges the light on a confocal spot in the sample 11a. Scanning of the excitation confocal spot is achieved by the scanning device 172, but scanning can also be achieved by translating the sample attached to multi-axis scanning stage. Axial scanning is achieved by scanning the objective lens 16 in its axial direction. The emitted light from the sample 11a is partially reflected on reflector 10, collected by the objective 16 and then

directed to the objective lens 17a as described in the first embodiment. A section plane is then selected (discussion below). The light is reflected on the mirror 12b and is then directed towards the detector 175. To enable the light to be properly spatially filtered by the pinhole 179, the mirror 12b and the scanning mirror 176 are adequately positioned. The light is filtered by the emission filter 178 and is then detected by the detector 175.

The invention is in general also directed to such an optical apparatus illustrated in Fig. 20, wherein the apparatus comprises only a first beam and wherein in the path of the first beam subsequently a beam splitter A (14a), a beam splitter B (14b), the quarter-wave plate (15a), the far focusing objective (17a) , the far focusing reflector (12b), the beam splitter B (14b), one or more means to direct light (12c, 12e, 176), beam splitter B (14b), beam splitter A (14a), an emission filter (178) and a pinhole (179) is present and wherein at least one of the means to direct light (176) is dynamically moveable. Preferably the pinhole can move in the perpendicular direction of the beam and the means to direct light is suitably a mirror that can be tilted as shown in the Figure. Preferably also the far focussing reflector (12b) is moveable such that it can move axially and be tilted.

The above apparatus is advantageous because it enables one to perform confocal microscopy, where the excitation and the detection are achieved from different angles, increasing the axial resolution of this kind of microscopy. The means to direct light which is dynamically moveable is preferably synchronized with the means to direct light in the excitation path (172).

FIG. 21 shows the apparatus 170 can be used to excite the sample as it is done in conventional confocal microscopy. FIG. 22 shows the apparatus 170 can also be used to excite the sample with an angle, by reflecting the excitation light with the reflector 10.

More importantly, the apparatus 170 enables detection in a novel way. While the sample 11a and its mirror images are conjugated in the objective lens 17a, the far focusing technique described in the first embodiment enables to detect the confocal emission in sample 11a under an angle, by selecting the proper section plane in sample 11b thanks to mirror 12b. The confocal

emission spot is then imaged on the pinhole 179 to enable final confocal detection.

FIG. 23 gives a schematic of how the detection is achieved thanks to the combination of the reflector 10 used in the sample 11a, the far focusing technique and the confocal detection. In the sample 11a, fluorescent emission is essentially reduced to a small region 201. This region has its mirror image 202 due to the reflector 10. These two regions are duplicated in the sample 11b thanks to refocusing. The imaging plane is selected at the center of the image of the region 202 by positioning mirror 12b. By centering this region on the pinhole 179 thanks to the scanning mirror 176, the emission is detected in a limited volume 203. Detection is therefore achieved with an arbitrary angle, preferably 90 degrees, compared to the excitation.

It will be seen that the present invention provides a method and apparatus for three-dimensional microscopy which provides live three-dimensional information, which has an enhanced axial resolution and which is not limited to fluorescence microscopy, but provides live three-dimensional information in bright field microscopy, dark-field microscopy, phase-contrast microscopy and differential-interference-contrast microscopy. Although the description above contains many specificities, these should not be construed as limiting, but as merely providing illustrations of some of the presently preferred embodiments of this invention. Thus, the scope of the invention should be determined by the appended claims and their legal equivalents.

The invention is thus also directed to the use of the optical apparatus according to the invention for live three-dimensional microscopy. Preferably by fluorescence microscopy, bright field microscopy, dark-field microscopy, phase-contrast microscopy and differential-interference-contrast microscopy or confocal microscopy.

CLAIMS

1. Optical apparatus comprising
a sample support and
imaging means arranged, when said optical apparatus is in use, to receive
a first beam emanating from the sample support under a first angle with respect to a reference axis
wherein the optical apparatus further comprises
a reflector and in that the imaging means are arranged to receive at least
a second beam emanating from the sample support under a second angle with respect to the reference axis,
the second angle being different from the first angle, the second beam when passing from the sample support to the imaging means being reflected by the reflector.

2. Optical apparatus according to claim 1, further comprising a beam splitter arranged to receive the first beam and the second beam and to split the first beam and the second beam between a first path and at least a different second path.

3. Optical apparatus according to claim 2, further comprising a beam combiner arranged to combine the first and second beam and direct the combined beam to the imaging means.

4. Optical apparatus according to claim 3, wherein beam splitter and beam combiner are one beam splitting and recombining means.

5. Optical apparatus according to any one of claims 1-4, wherein the apparatus further comprises a main objective lens and wherein the sample support is positioned between the objective lens and the reflector.

6. Optical apparatus according to any one of claims 2-5, wherein the apparatus further comprises in the path of the first and optional second beam a far focusing reflector combined with an optional far focusing objective, the far focusing reflector facing the front lens of said far focusing objective lens and a quarter-wave plate and a beam splitter means positioned before the far focusing reflector or before the far focusing objective if present.

7. Optical apparatus according to claim 6, wherein the apparatus further comprises rotation means to rotate the quarter-wave plate.

8. Optical apparatus according to any one of claims 6-7, wherein the apparatus further comprises in the path of the first and optional second beam one or more lenses to conjugate appropriately the image of the sample toward the far focusing objective lens.

9. Optical apparatus according to any one of claims 6-9, wherein the imaging means for detecting and recording images is positioned to detect and record said observed light, said observed light having been split by beam splitting and recombining means, far focused by the optional far focusing objective lens or lenses and combined by the beam splitting and recombining means.

10. Optical apparatus according to any one of claims 6-9, wherein the apparatus further comprises a plurality of means to direct light from the sample support to the beam splitting and recombining means as well to and from the one or two optional far focusing objective lenses and towards said imaging means.

11. Optical apparatus according to any one of claims 6-10, wherein apparatus comprises only a first beam and wherein in the path of the first beam a beam splitter A, a beam splitter B, the quarter-wave plate, the optional far focusing objective, the far focusing reflector, the beam splitter B, one or more means to direct light, beam splitter B, beam

splitter A , an emission filter and a pinhole is present and wherein at least one of the means to direct light is dynamically moveable.

12. Optical apparatus according to any one of claims 1-10, wherein the apparatus further comprises active or passive means to modify phase and amplitude of the beams emanating from the sample.

13. Optical apparatus according to claim 12, wherein a beam combiner is arranged to combine the first and second beam and direct the combined beam to the imaging means and wherein in the path of the combined beam to the imaging means a spatial light modulator and two lenses a and b are positioned, wherein lens a is positioned so that its back focal plane coincides with far focusing objective back focal plane, wherein the spatial light modulator is positioned in lens a front focal plane and wherein lens b back focal plane is positioned on the spatial light modulator to image the two different planes selected by the two far focusing objective lenses side by side on the imaging means.

14. Optical apparatus according to any one of claims 1-13, wherein the apparatus further comprises means to visualize the said microscope sample.

15. Optical apparatus according to any one of claims 1-14, wherein the apparatus further comprises focusing and imaging means.

16. Optical apparatus according to claim 15, wherein the apparatus further comprises means for positioning the focusing and imaging means.

17. Optical apparatus according to any one of claims 1-16, wherein the apparatus further comprises means to adjust position and orientation of the reflector relative to the sample support.

18. Optical apparatus according to claim 17, wherein the apparatus further comprises means for sensing position and orientation of the reflector relative to the sample support.

19. Optical apparatus according to any one of claims 1-18, wherein the apparatus further comprises rotation means to rotate the sample support relative to the reflector or to rotate the reflector relative to the sample support.

20. Optical apparatus according to any one of claims 5-19, wherein the apparatus further comprises means to adjust position of said main objective lens relative to the sample support.

21. Optical apparatus according to claim 20, wherein the apparatus further comprises means for sensing position of the main objective lens relative to the sample support.

22. Optical apparatus according to claim 21, wherein the means to adjust position is responsive to the means for sensing position.

23. Optical apparatus according to any one of claims 6-22, wherein the apparatus further comprises means to adjust position of the far focusing objective lens relative to the far focusing reflector provided a far focusing objective is present.

24. Optical apparatus according to any one of claims 6-23, wherein the apparatus further comprises means to adjust position of the far focusing reflector relative to the far focusing objective lens provided a far focusing objective is present.

25. Optical apparatus according to claim 24, wherein the apparatus further comprises means for sensing position of the far focusing reflector relative to the far focusing objective lens provided a far focusing objective is present.

26. Optical apparatus according to claim 25, wherein means to adjust position is responsive to the means for sensing position.

27. Optical apparatus according to any one of claims 5-26, wherein the apparatus further comprises sample illumination means for providing illuminating light to the sample support, the illuminating light positioned to provide illuminating light to the main objective lens.

28. Optical apparatus according to any one of claims 1-27, wherein the apparatus further comprises sample side illumination means for providing side illuminating light to the sample support, the side illuminating light positioned to provide illuminating light to the reflector and the sample support.

29. Optical apparatus according to claim 28, wherein the apparatus further comprises selective transmittance and reflectance means for transmitting the light from the main objective lens toward the imaging means and reflecting said illuminating light away from said imaging means.

30. Optical apparatus according to 29, wherein the apparatus further comprises filtering means for transmitting the observed light from the main objective lens and filtering the illuminating light from the illuminating means.

31. Optical apparatus according claim 29, wherein the apparatus further comprises selective transmittance and reflectance means for reflecting the observed light from the main objective lens toward the imaging means and transmitting the illuminating light away from said imaging means.

32. Optical apparatus according to any one of claims 1-31, wherein the reflector is a planar mirror.

33. Optical apparatus according to any one of claims 1-31, wherein the reflector is a glass substrate presenting a repeated structure and coated with a dielectric or a metallic layer.

34. Optical apparatus according to any one of claims 1-31, wherein the reflector is a substrate presenting a single dihedral structure and coated with a dielectric or a metallic layer.

35. Optical apparatus according to claim 34, wherein the reflector is a glass, plastic or crystalline substrate presenting a repeated structure and optionally coated with a dielectric or a metallic layer and wherein the substrate comprises more than one dihedral structure, which can cross and whose crossing orientation in respect to each other can range from 0 to 90 degrees.

36. Optical apparatus according to any one of claims 1-35, wherein the apparatus further comprises a microfluidic device comprising at least one flow channel obtained by standard soft lithography with a polymer layer, preferably made of low refractive index optical adhesives or coating materials or poly(dimethylsiloxane) (PDMS).

37. A method for three-dimensional optical microscopy of a microscope sample using the optical apparatus according to any one of claims 6-36 wherein at least one far focusing objective lenses is present and wherein the sample is positioned in the sample support comprising the steps of:

(a) focusing said the main objective lens about the said microscope sample;

(b) oscillating the focus of the far focusing objective lenses upon a volume within one of the images of the said sample;

(c) directing the images observed by said far focusing objectives to the image detection means for image recording.

38. A method for three-dimensional optical microscopy of a microscope sample using the optical apparatus according to any one of claims 6-36 wherein at least one far focusing objective lenses is present and wherein the sample is positioned in the sample support and comprising the steps of:

(a) focusing said the main objective lens about the said microscope sample;

(b) focusing the far focusing objective lenses upon a section or plane within the image of the said sample;

(c) positioning adequately the active or passive means to modify phase and amplitude of the observed light in order to achieve dark field microscopy, phase microscopy, differential interference contrast microscopy, confocal microscopy or to extend the depth of field of the image of the said sample;

39. A method for three-dimensional optical microscopy of a microscope sample using the optical apparatus according to any one of claims 6-36 wherein at least one far focusing objective lenses is present and wherein the sample is positioned in the sample support comprising the steps of:

(a) focusing said the main objective lens about the said microscope sample;

(b) focusing the far focusing objective lenses upon a section or plane within the image of the said sample;

(c) directing images observed by said far focusing objectives to the image detection means for image recording;

(d) adjusting said far focusing mirrors facing said far focusing objectives to enable imaging of a new set of volume sections;

(e) repeating steps (a), (b), (c) and (d) until a plurality of section of said sample has been observed and recorded, forming a data set of recorded images;

(f) processing data set obtained at step (e) to extract the images of the said microscope sample obtained with different orientations;

(g) reorienting the said images obtained at step (f) in the same base;

(h) combining the images obtained at step (g) to create a new volume image of the sample with enhanced resolution.

40. Optical method to image a sample using an imaging means which use a first beam emanating from said sample under a first angle with respect to a reference axis and a second beam emanating from said sample under a second angle with respect to said reference axis to image the sample and wherein this second angle is different than the first angle and wherein the second beam is reflected when passing from the sample to the imaging means.

41. Optical method according to claim 40, wherein the imaging means are suited for fluorescence microscopy, bright field microscopy, dark-field microscopy, phase-contrast microscopy, differential-interference-contrast microscopy or confocal microscopy.

42. Use of the optical apparatus according to any one of claims 1-36 for live three-dimensional microscopy.

43. Use according to claim 42, wherein the microscopy is fluorescence microscopy, bright field microscopy, dark-field microscopy, phase-contrast microscopy, differential-interference-contrast microscopy or confocal microscopy.

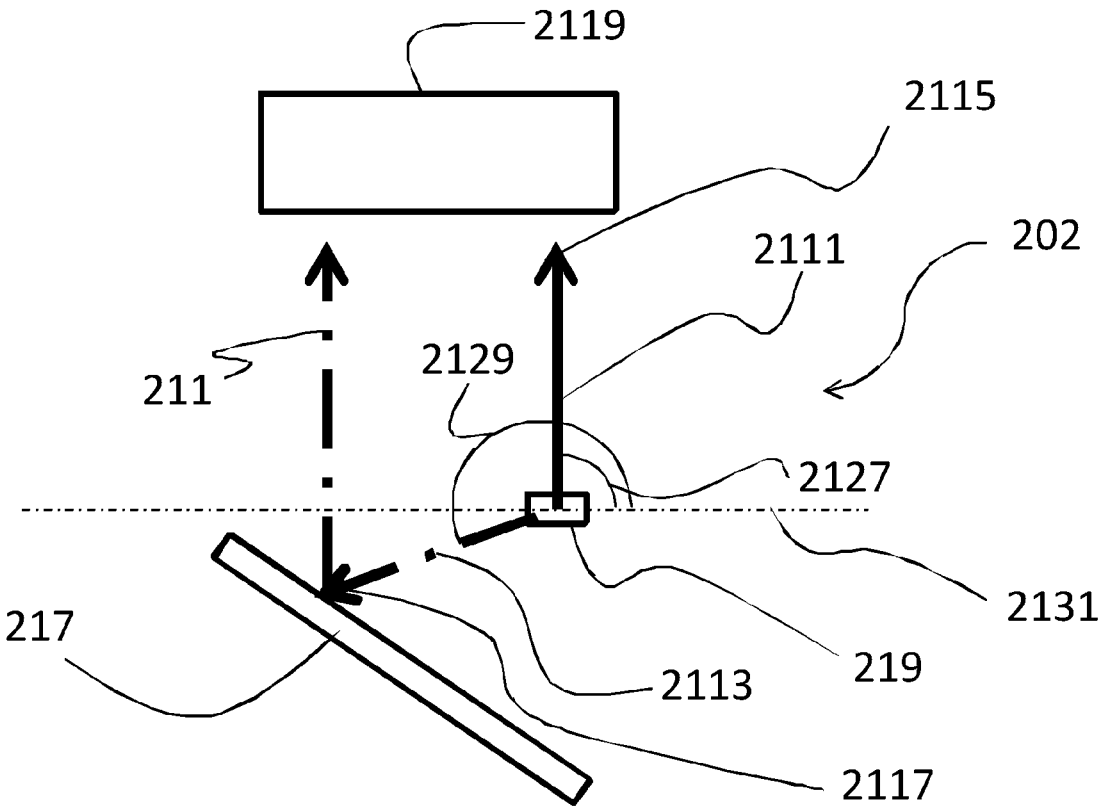


Fig.1

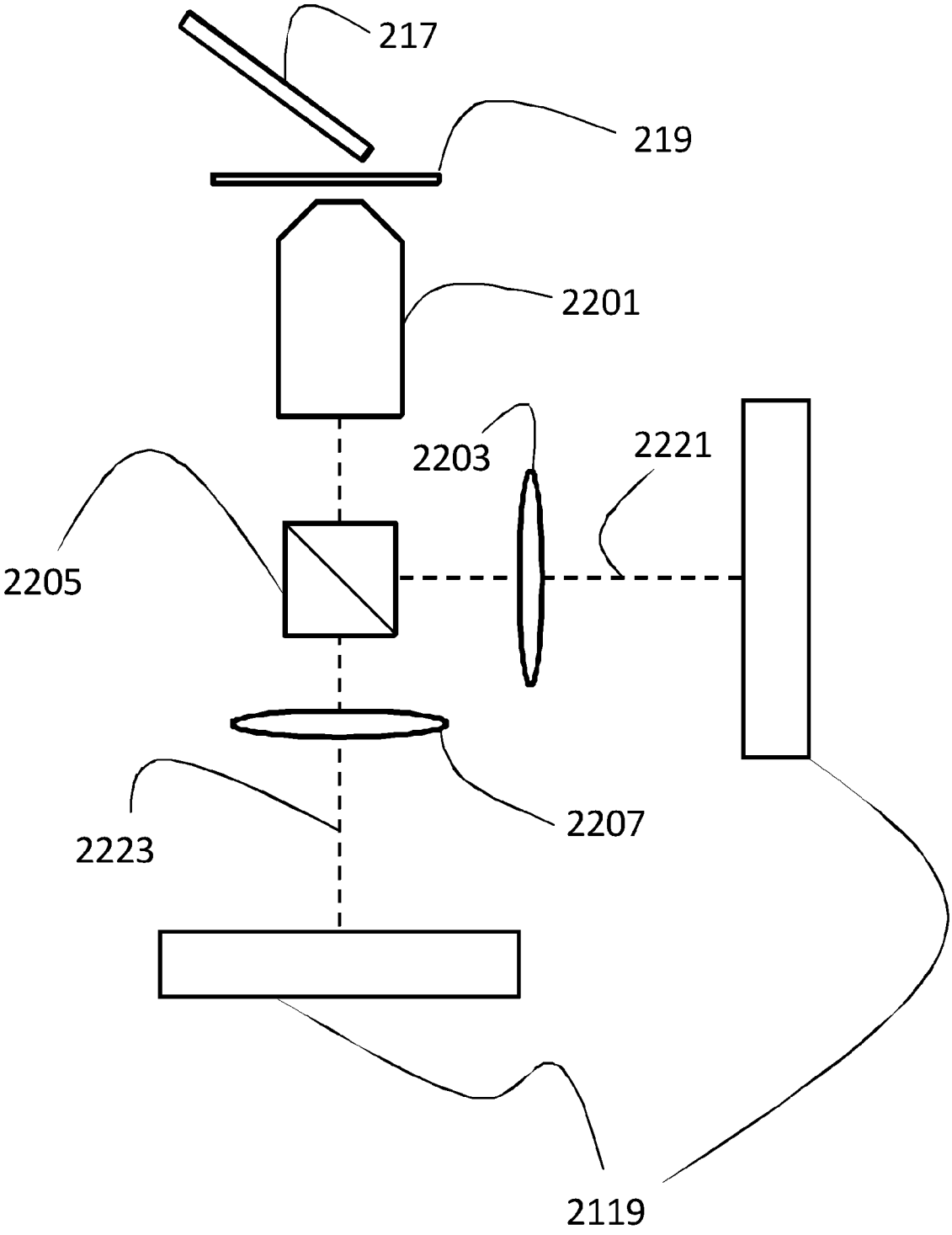


Fig.2

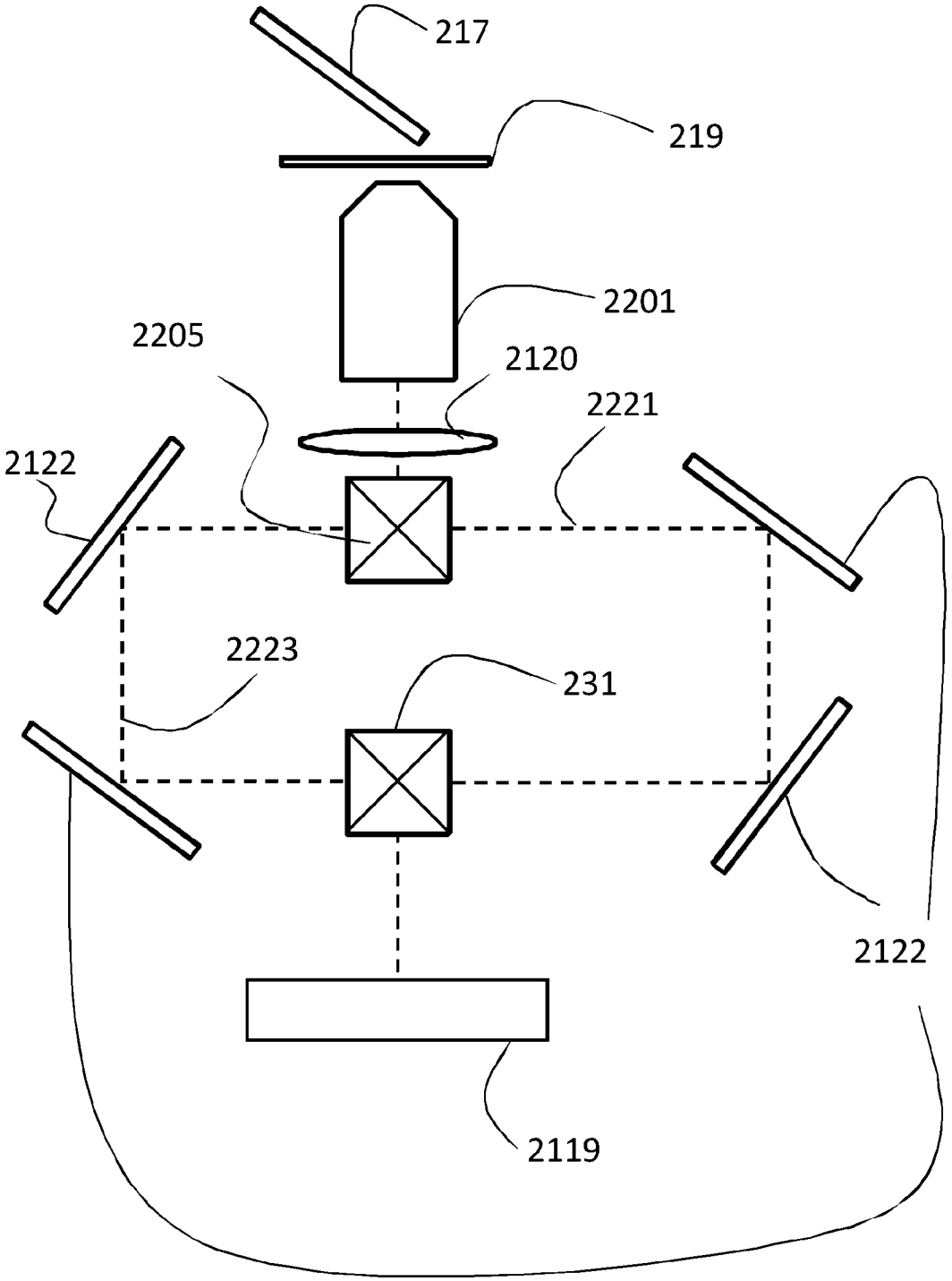


Fig.3

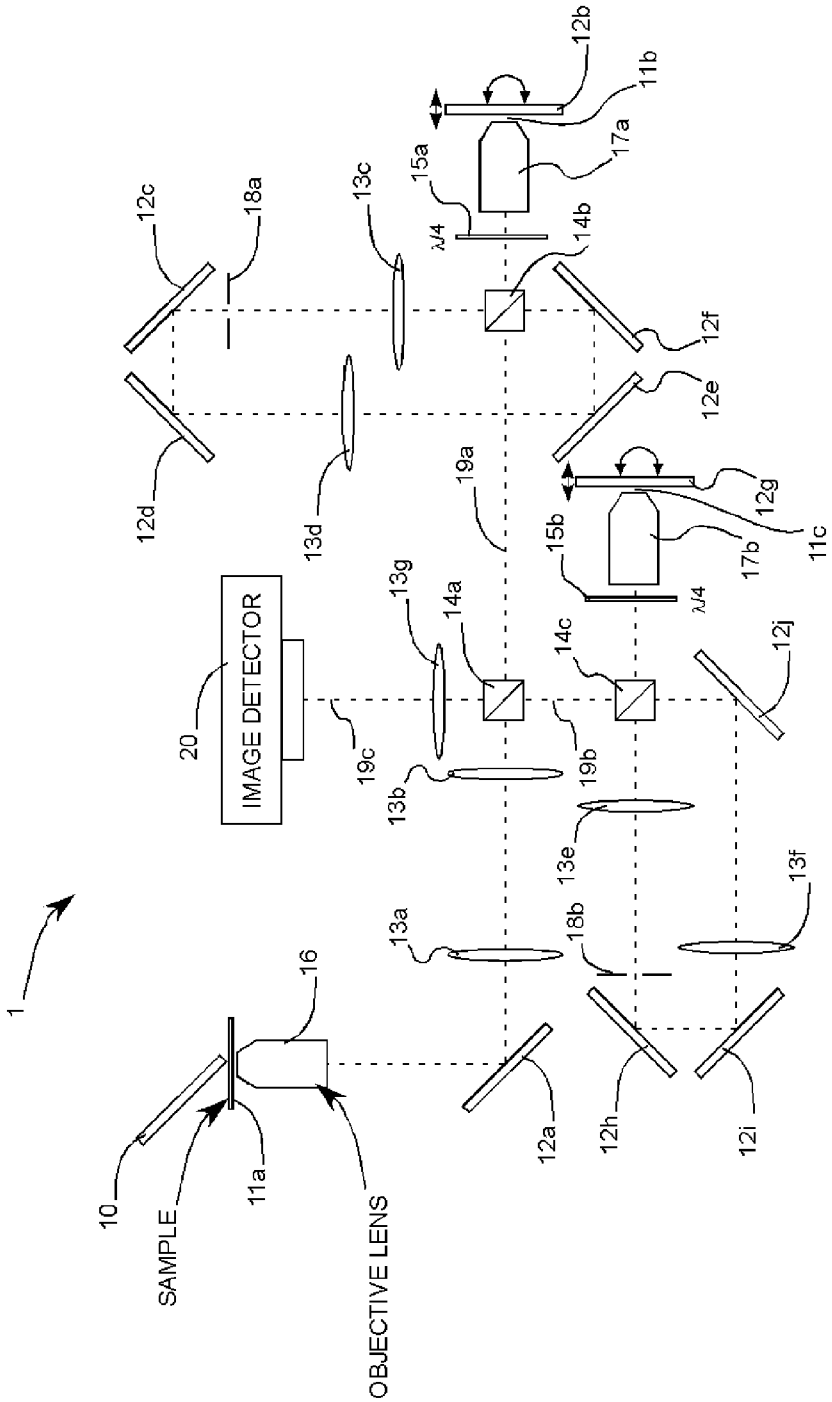

1
10
SAMPLE
11a
16
OBJECTIVE LENS
12a
13a
18b
12h
12i
13f
12j
13e
19b
14c
19c
13b
13g
14a
20
IMAGE DETECTOR
19a
15b
λ/4
17b
12g
11c
12d
13d
12e
12f
14b
12c
18a
13c
15a
λ/4
17a
11b
12b

Fig. 4

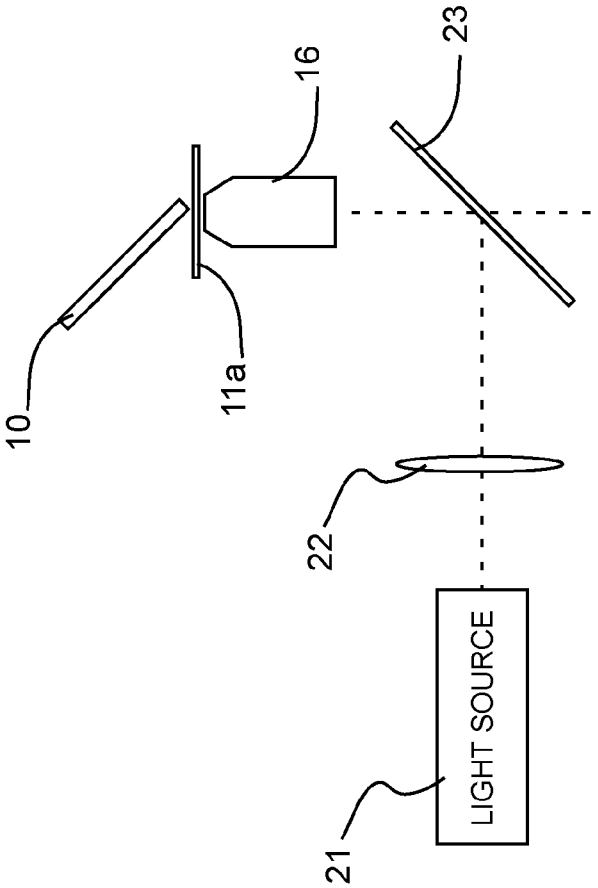


Fig. 5

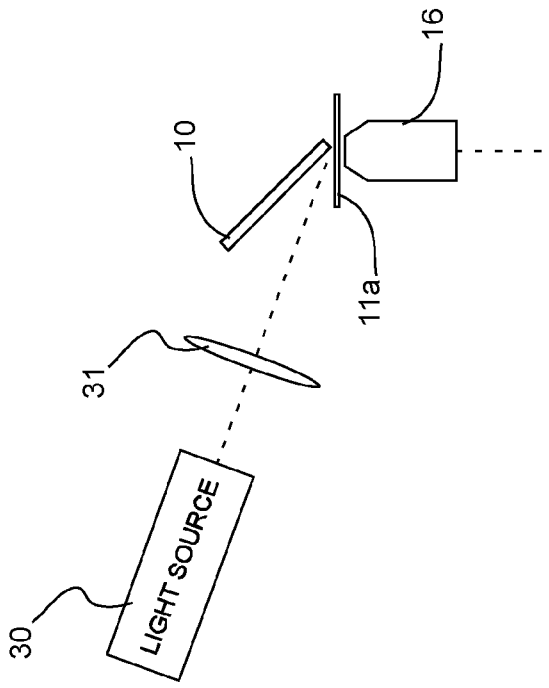


Fig.6

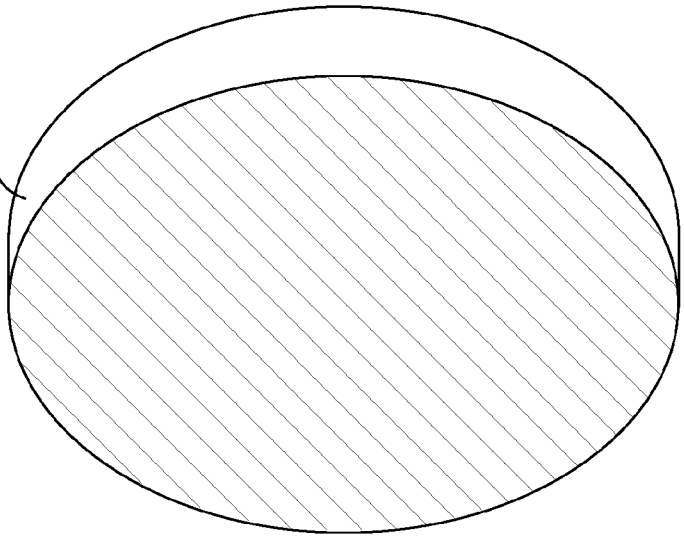


Fig.7

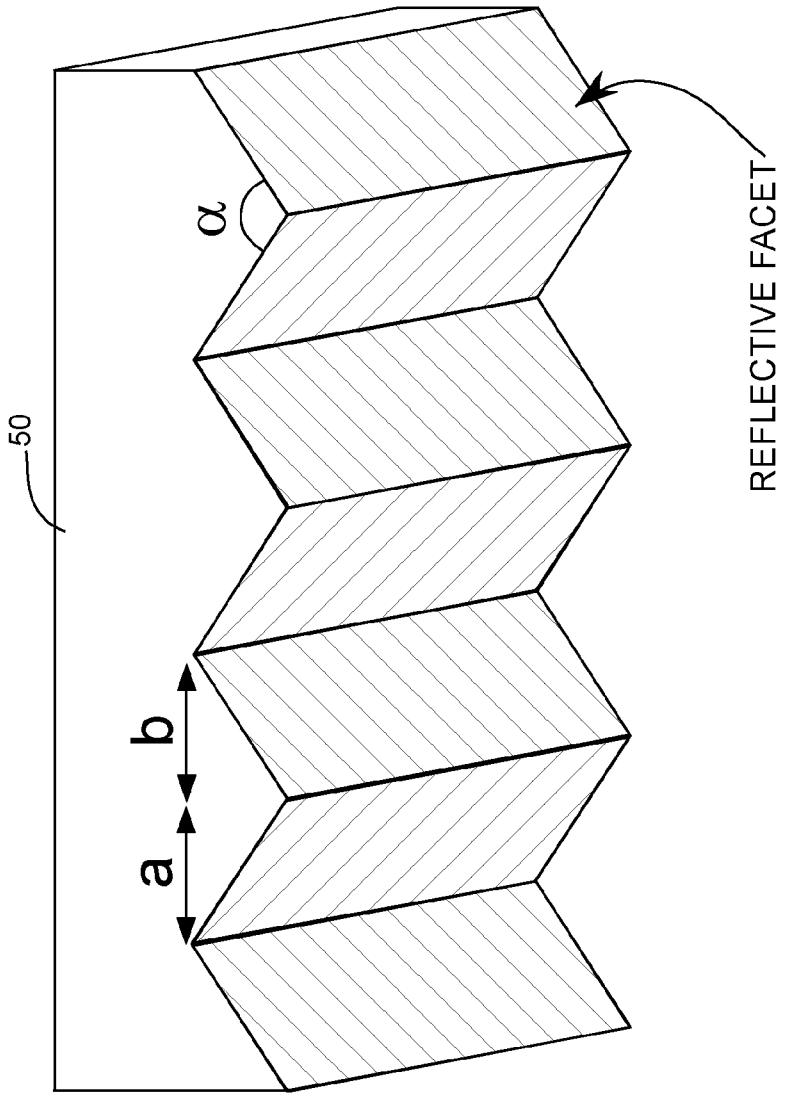


Fig. 8

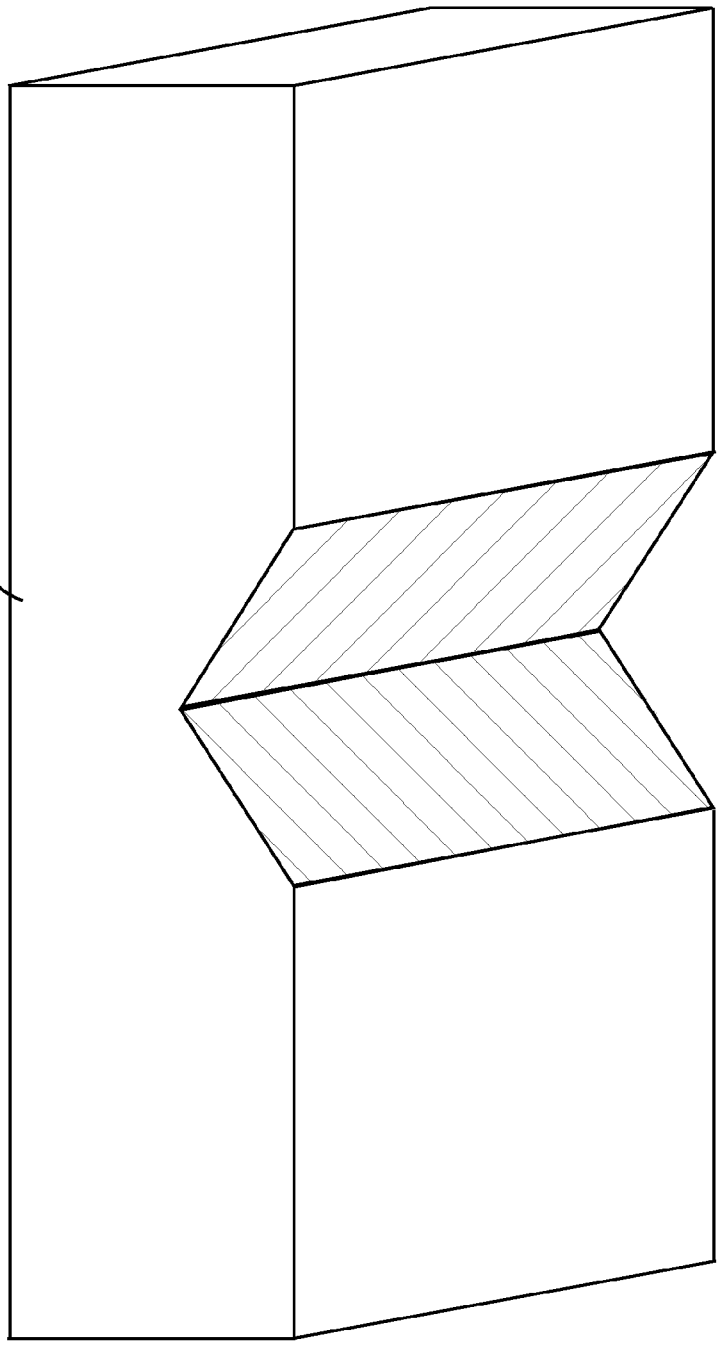


Fig.9

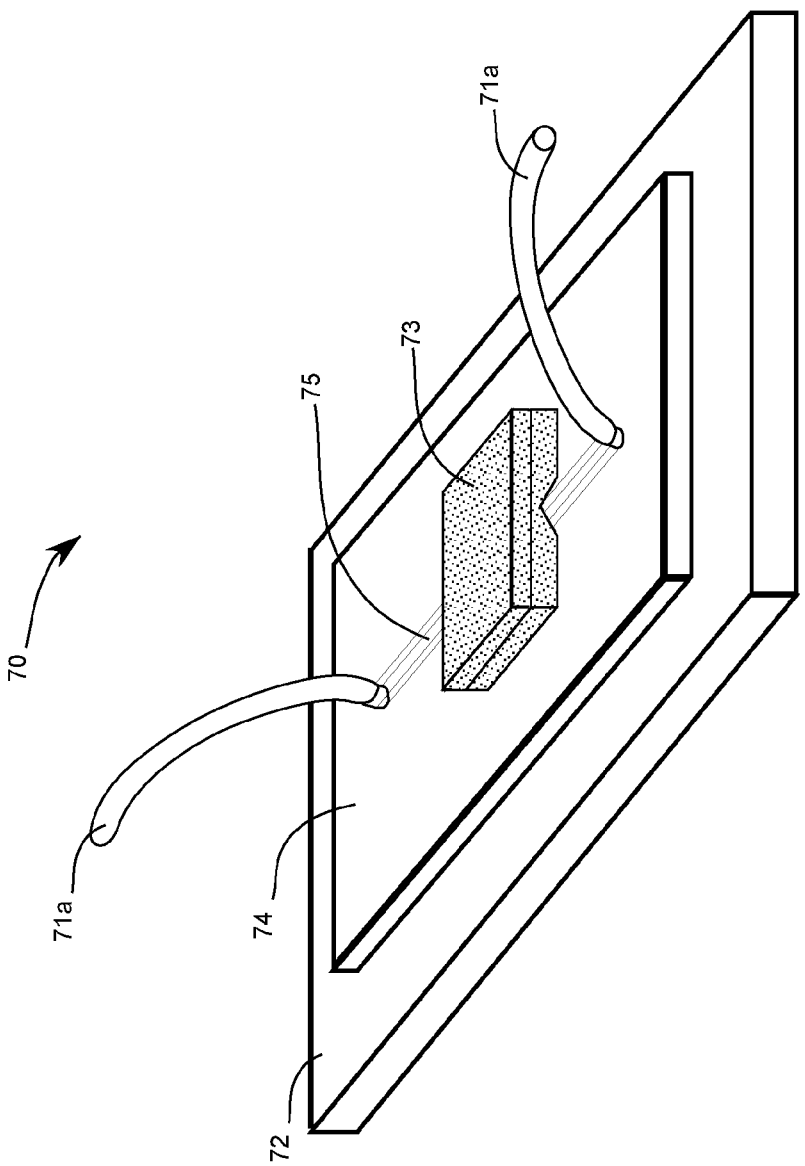


Fig. 10

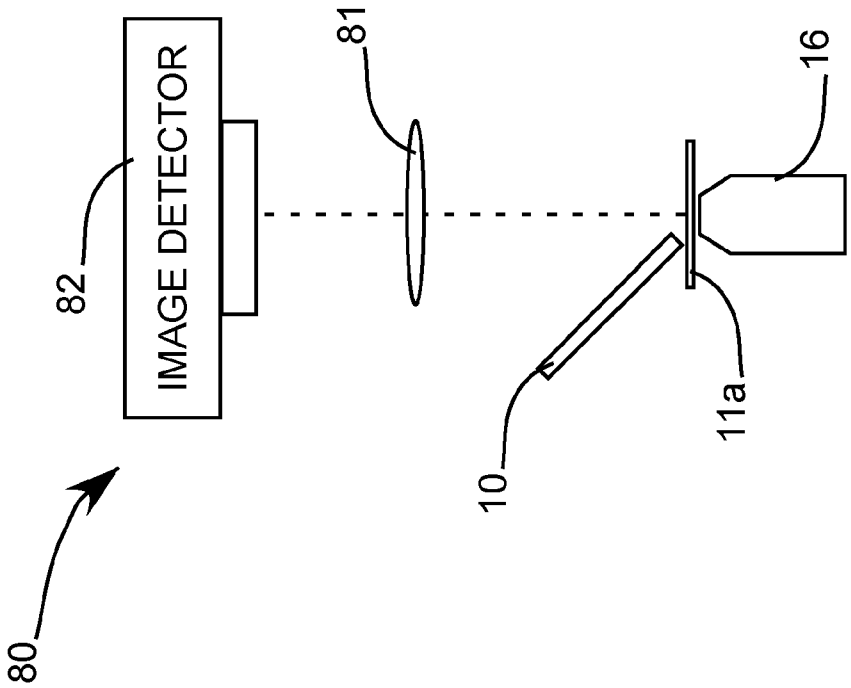
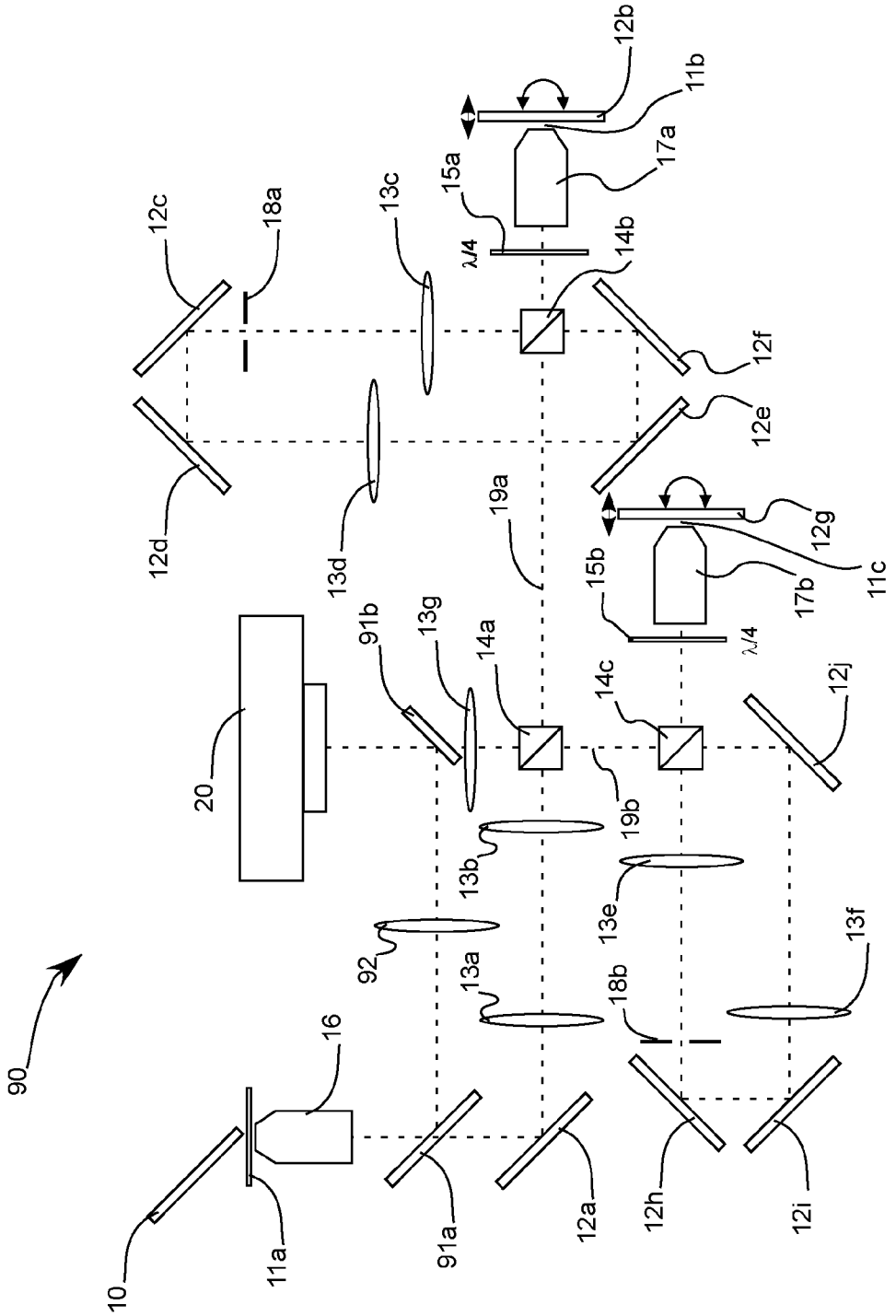


Fig. 11


90
10
11a
16
91a
12a
92
13a
13b
20
91b
13g
14a
19b
14c
13e
18b
12h
13f
12i
12j
19a
15b
λ/4
17b
12g
11c
13d
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12e
12f
14b
15a
λ/4
17a
11b
12b
13c
18a
12c

Fig. 12

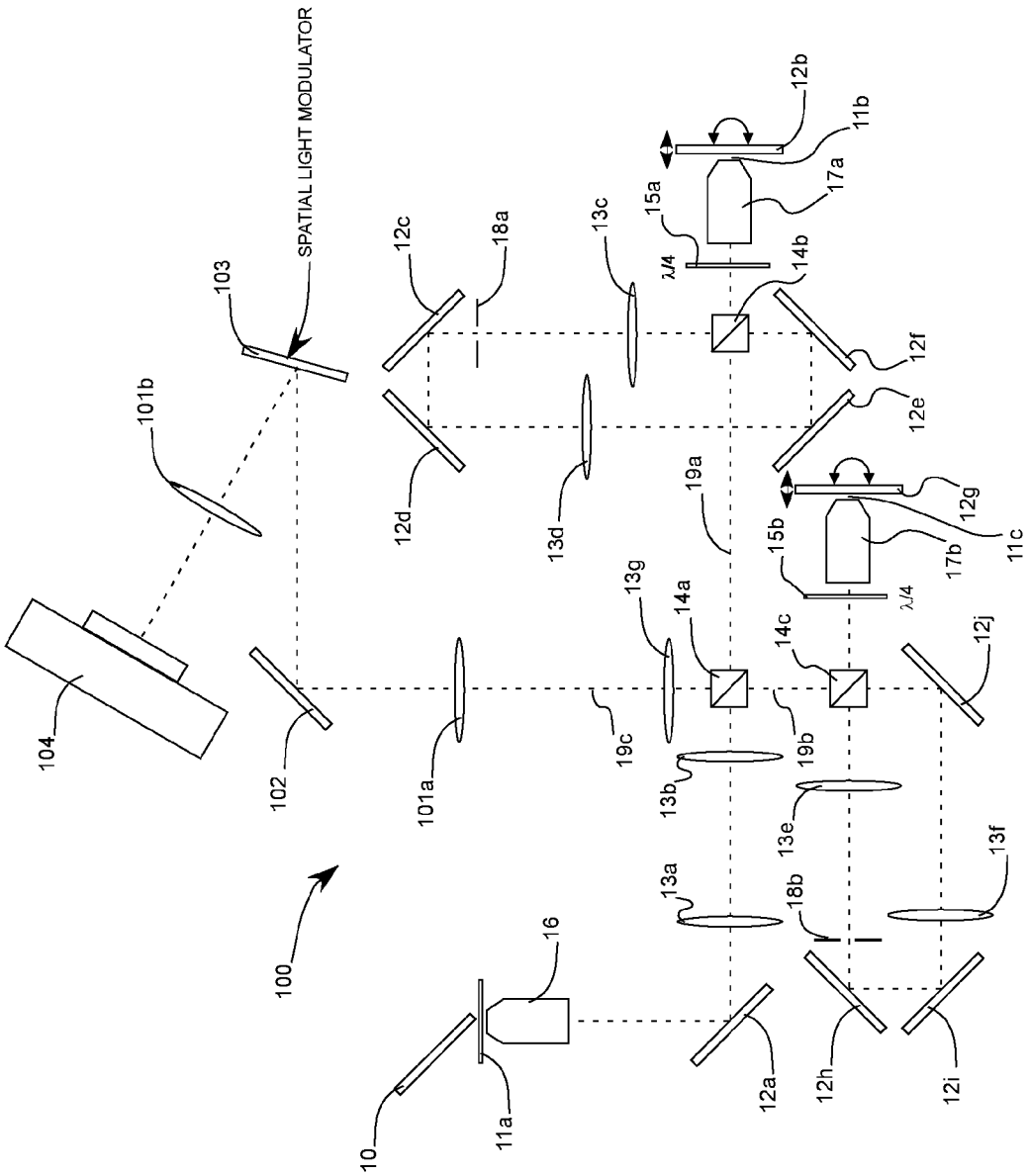
SPATIAL LIGHT MODULATOR
103
101b
104
102
101a
100
10
11a
16
12a
12h
12i
12j
13a
13b
13e
13f
19c
19b
13g
14a
14c
18b
19a
13d
12d
12c
18a
13c
15a
λ/4
12b
11b
17a
14b
12f
12e
15b
λ/4
17b
12g
11c

Fig. 13

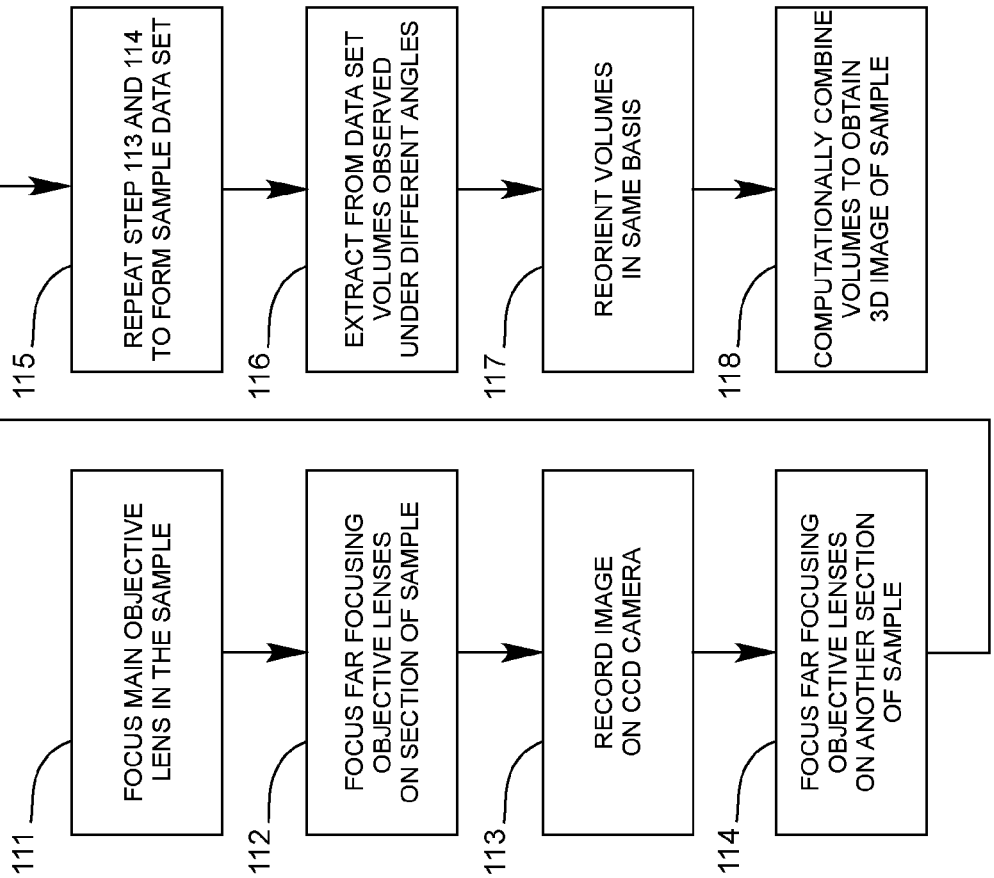
111
FOCUS MAIN OBJECTIVE LENS IN THE SAMPLE
112
FOCUS FAR FOCUSING OBJECTIVE LENSES ON SECTION OF SAMPLE
113
RECORD IMAGE ON CCD CAMERA
114
FOCUS FAR FOCUSING OBJECTIVE LENSES ON ANOTHER SECTION OF SAMPLE
115
REPEAT STEP 113 AND 114 TO FORM SAMPLE DATA SET
116
EXTRACT FROM DATA SET VOLUMES OBSERVED UNDER DIFFERENT ANGLES
117
REORIENT VOLUMES IN SAME BASIS
118
COMPUTATIONALLY COMBINE VOLUMES TO OBTAIN 3D IMAGE OF SAMPLE

Fig. 14

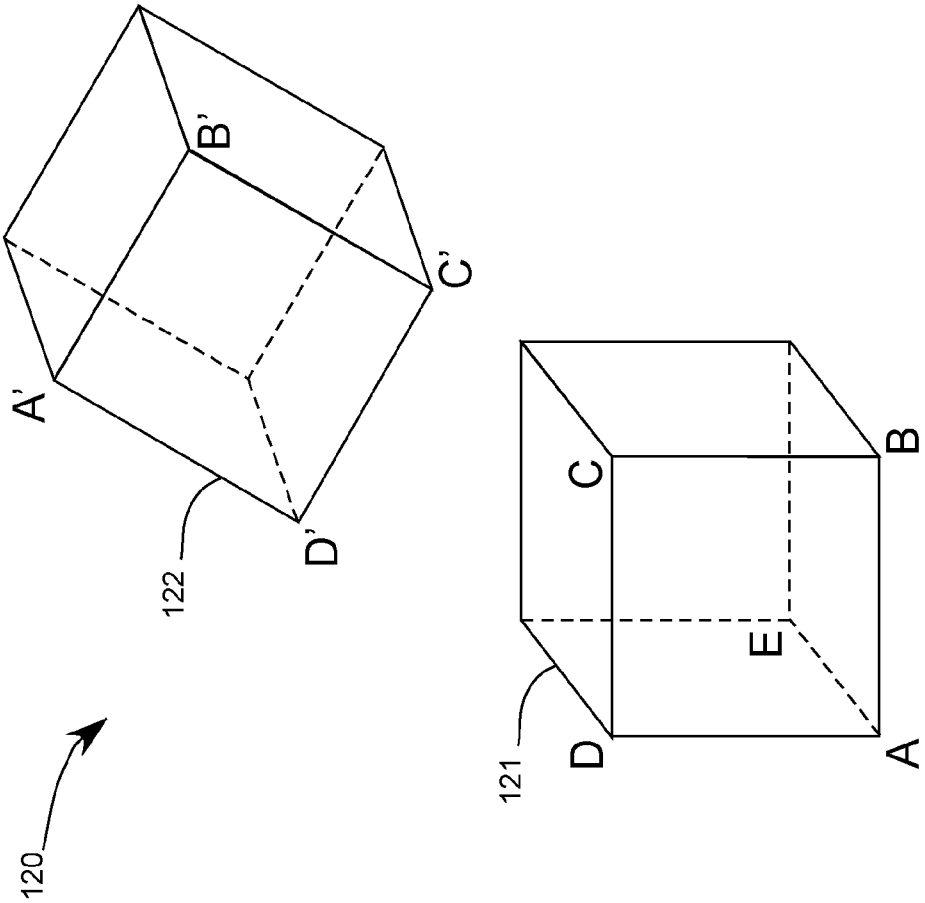

Fig. 15

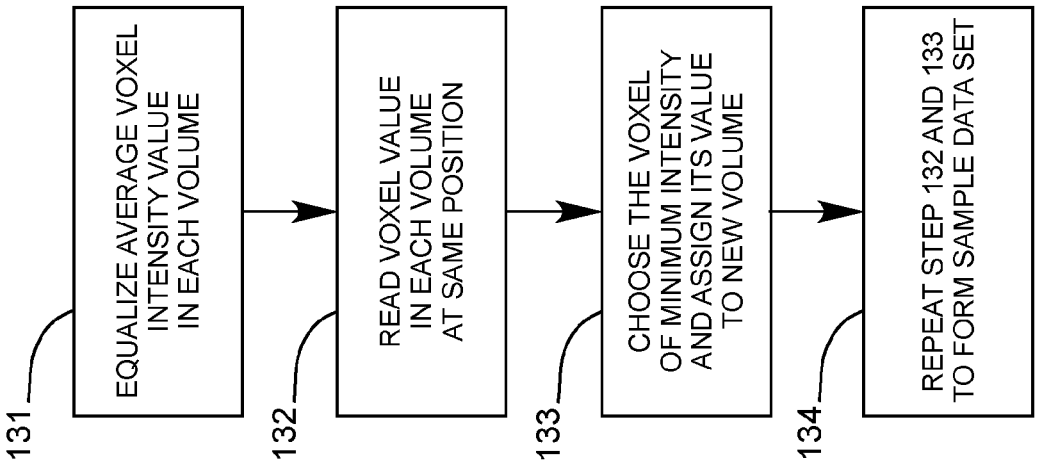
131
EQUALIZE AVERAGE VOXEL INTENSITY VALUE IN EACH VOLUME
132
READ VOXEL VALUE IN EACH VOLUME AT SAME POSITION
133
CHOOSE THE VOXEL OF MINIMUM INTENSITY AND ASSIGN ITS VALUE TO NEW VOLUME
134
REPEAT STEP 132 AND 133 TO FORM SAMPLE DATA SET

Fig. 16

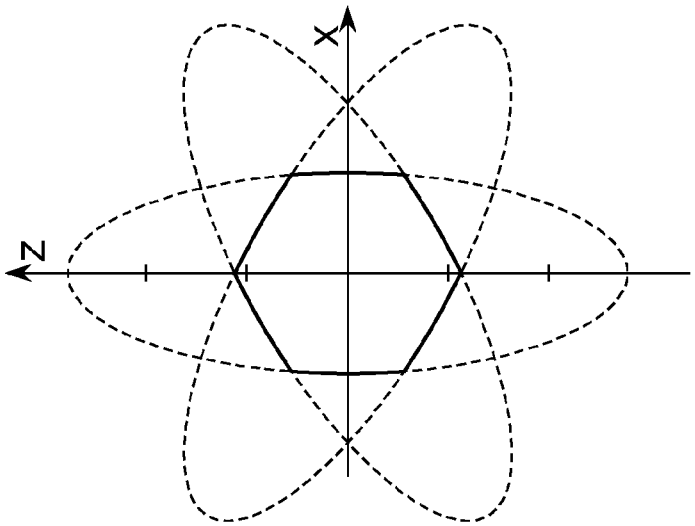

Fig.18

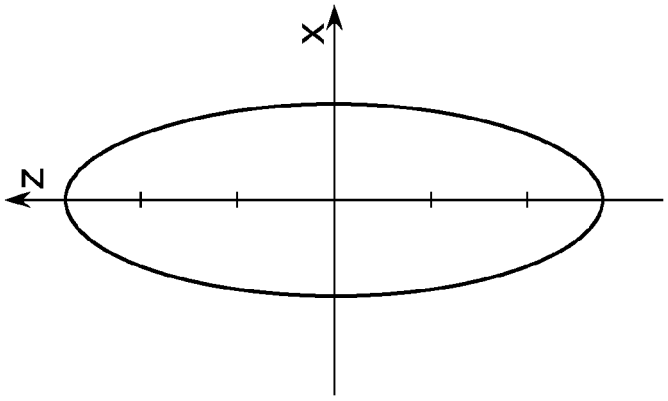

Fig. 17 (PRIOR ART)

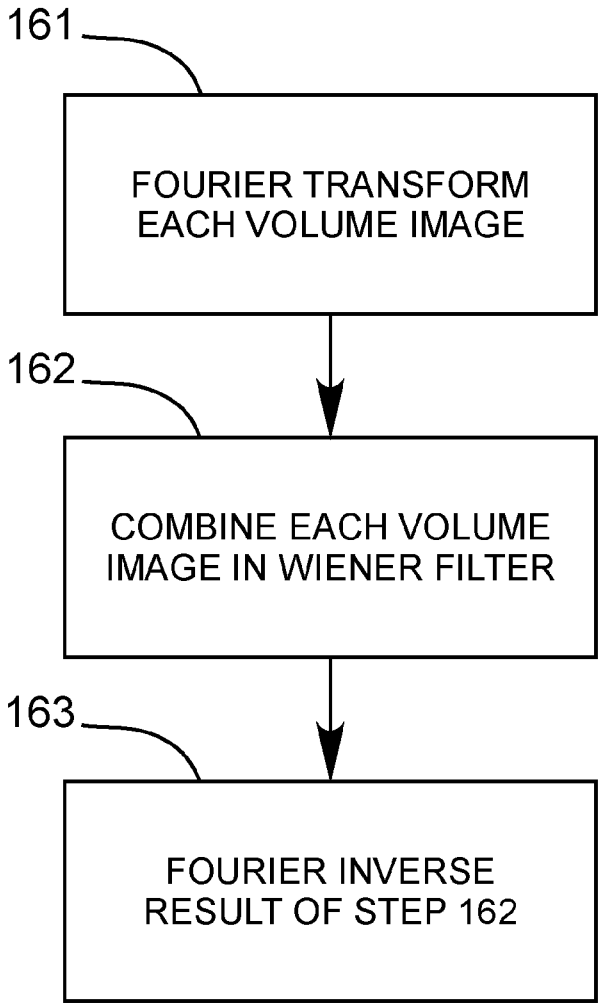
161
FOURIER TRANSFORM EACH VOLUME IMAGE
162
COMBINE EACH VOLUME IMAGE IN WIENER FILTER
163
FOURIER INVERSE RESULT OF STEP 162

Fig. 19

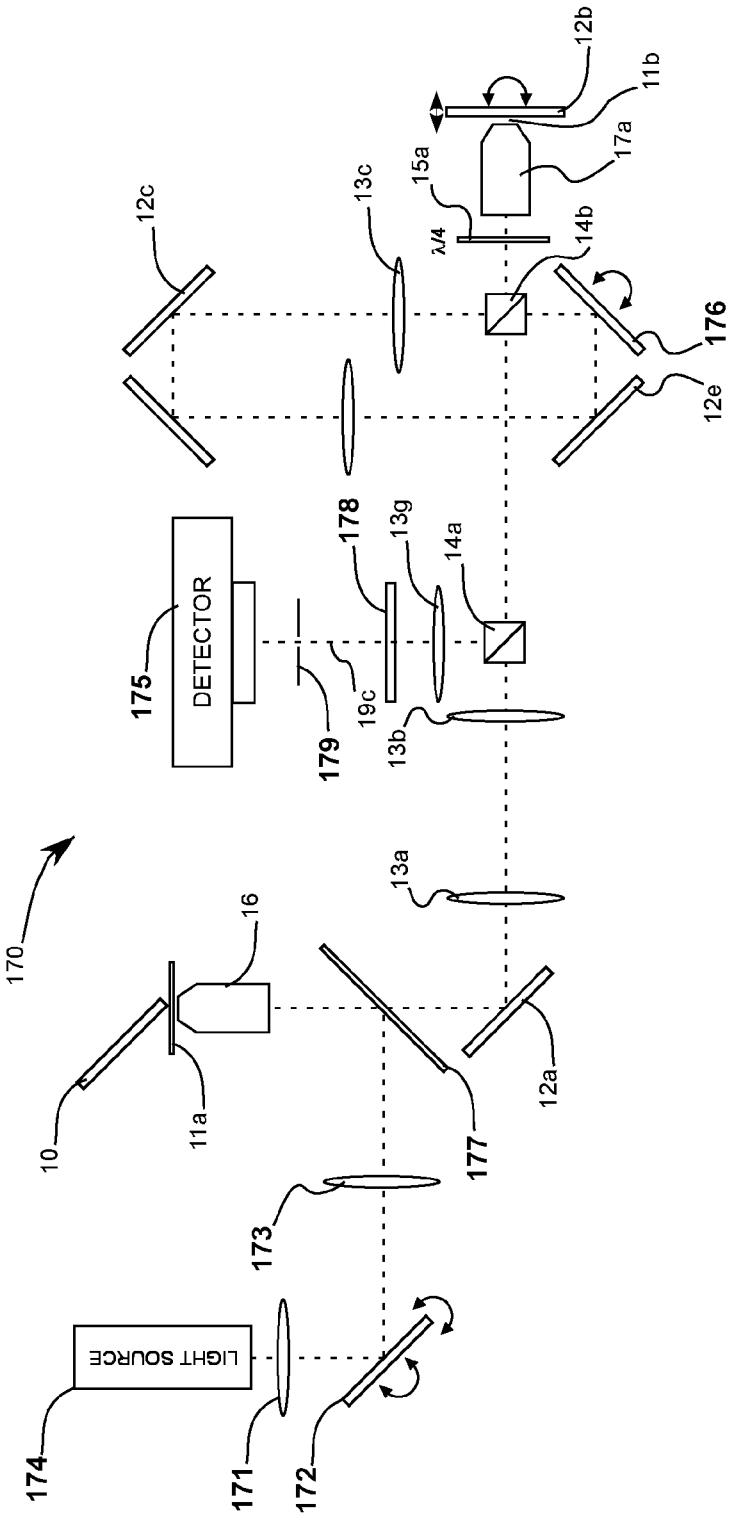

Fig. 20

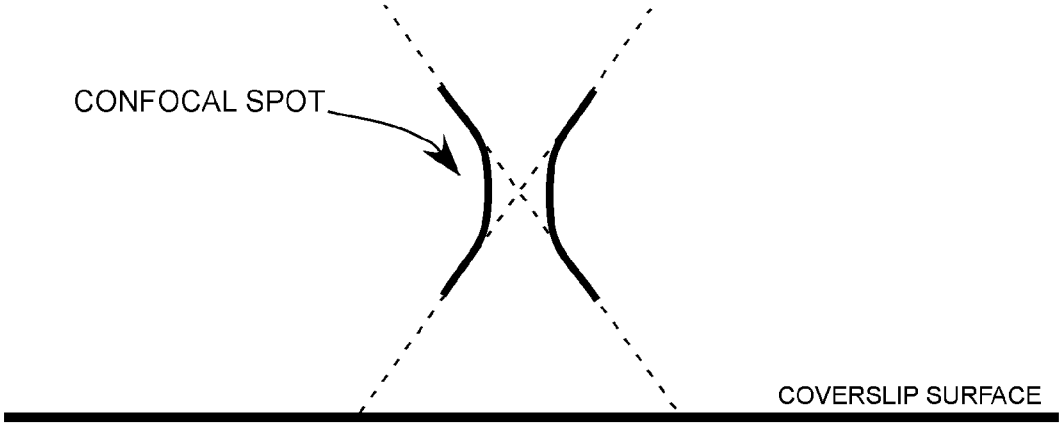

Fig. 21 (PRIOR ART)

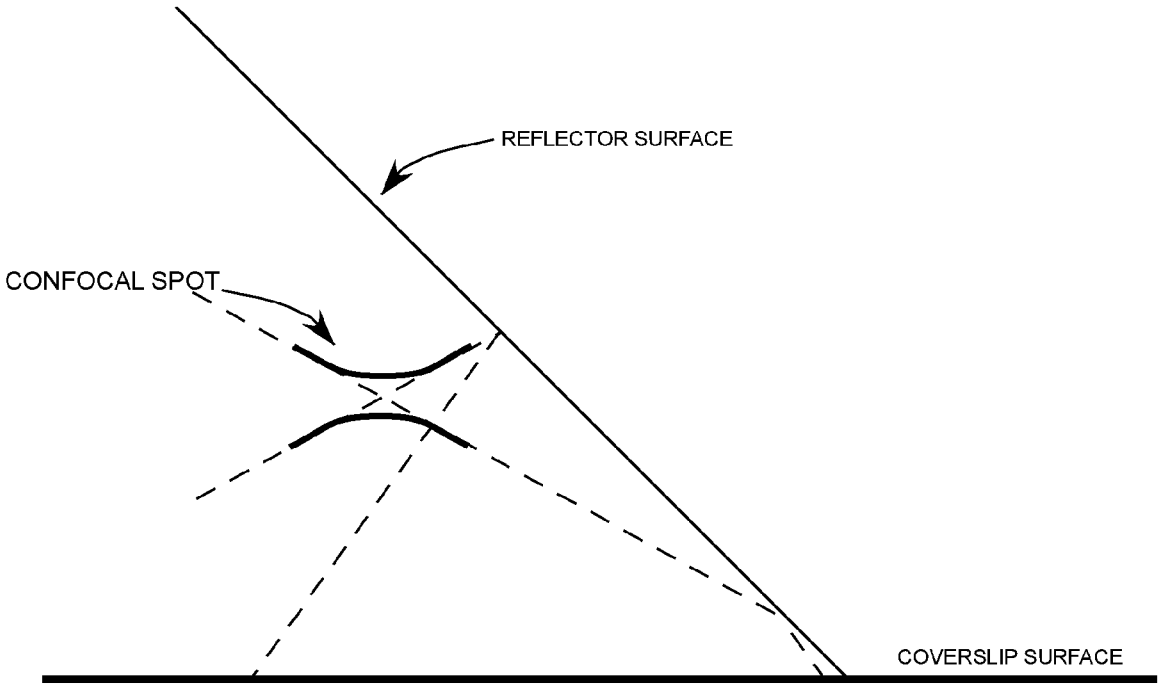

Fig. 22

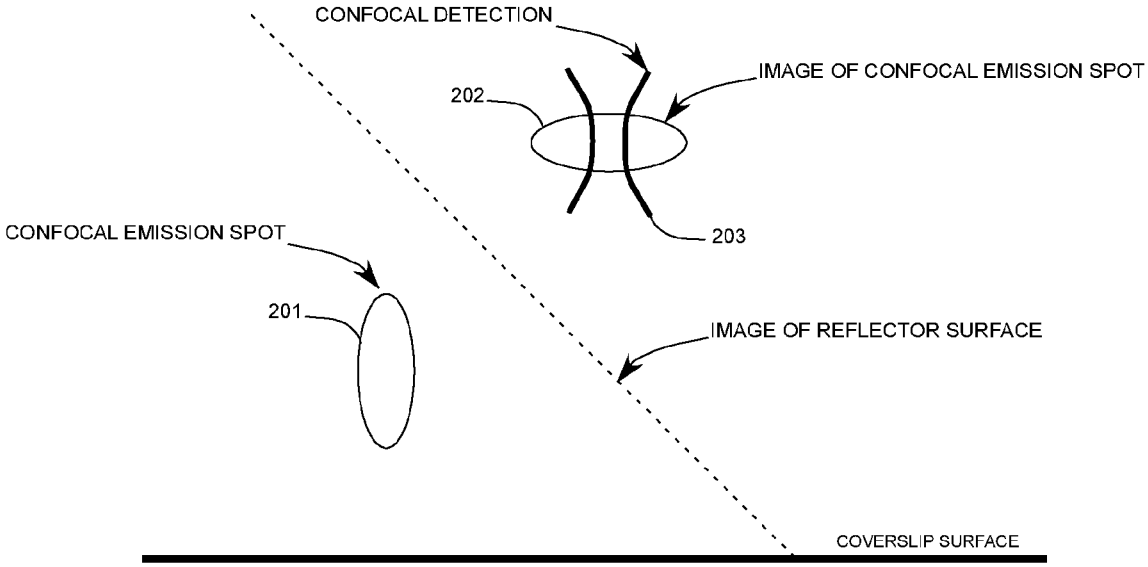
CONFOCAL DETECTION
IMAGE OF CONFOCAL EMISSION SPOT
202
203
CONFOCAL EMISSION SPOT
201
IMAGE OF REFLECTOR SURFACE
COVERSLIP SURFACE

Fig. 23

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2013/050378

A. CLASSIFICATION OF SUBJECT MATTER
INV. G02B21/22 G02B21/36
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G02B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2005/128287 A1 (HANZAWA TOYOHARU [JP]) 16 June 2005 (2005-06-16) paragraph [0061] - paragraph [0084]; figure 6	1-43
A	DE 102 50 953 A1 (ZEISS CARL [DE]) 19 May 2004 (2004-05-19) abstract; figure 1	1,40
A	DE 10 2010 015691 A1 (ZEISS CARL MICROIMAGING GMBH [DE]) 27 October 2011 (2011-10-27) abstract; figure 1	1,40
A	KR 2005 0011634 A (CHOI HAE YONG) 29 January 2005 (2005-01-29) abstract	1,40

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 July 2013	09/07/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lehtiniemi, Henry

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2013/050378

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE 10 2006 009452 A1 (ZEISS MEDITEC AG CARL) 26 April 2007 (2007-04-26) abstract; figure 1A -----	1,40

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2013/050378

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2005128287 A1	16-06-2005	JP 4508569 B2	21-07-2010
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KR 20050011634 A	29-01-2005	NONE	
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		US 2008266657 A1	30-10-2008
		US 2011170179 A1	14-07-2011
		WO 2007045499 A1	26-04-2007